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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Extensive studies have demonstrated that the Akt pathway is essential for cell survival and anti-apoptosis; however, alterations of Akt in human malignancy have not been documented. We have recently demonstrated significantly increased AKT1 and AKT2 kinase activity in primary ovarian carcinomas. We have also shown that PI3K is frequently activated in the specimens with activation of Akt. The majority of cases with PI3K/Akt activation are late stage and high grade. The biological significance of AKT1 activation in human cancer was demonstrated by malignant transformation of NIH 3T3 cells transfected with constitutively active AKT1, but not cells transfected with wild type AKT1. Moreover, inhibition of PI3K/Akt pathway inhibits cell growth and induces apoptosis in human ovarian cancer cell lines. We have also observed that estrogen receptor (ER)α interacts and activates PI3K/Akt pathway. PI3K/Akt feedback regulates ERα by phosphorylation of serine-167 of ERα. These data indicate that AKT1 kinase, which is frequently activated in human cancer, is a determinant in oncogenesis and a potential target for cancer intervention.

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INTRODUCTION:

The purpose of this project is to: 1) Determine the incidence and clinical significance of *PI3K/AKT1* alterations in ovarian cancer; 2) Determine the role of overexpression of active and wild type PI3K and AKT1 in ovarian surface epithelial cell transformation and 3) Determine PI3K and AKT1 as targets for ovarian cancer intervention

BODY:

During the current budget year, we focused our efforts on the examination of incidence and clinical significance of PI3K/AKT1 alterations in human ovarian cancer and PI3K/AKT1 as a potential target for ovarian cancer intervention.

I. PI3K/AKT1 is frequently activated in human primary ovarian cancer.

Extensive studies have demonstrated that the Akt pathway is essential for cell survival and anti-apoptosis (1); however, alterations of Akt in human malignancy have not been documented. Total 91 ovarian tumor specimens have been examined for protein expression and kinase activity of AKT1. Activation of PI3K and AKT1 was observed in 39.3% tumor examined, however, elevated AKT1 protein level was only detected in 8.3% (2, see Appendix). We have also looked at PTEN, a tumor suppressor gene encoding dual phosphatase that dephosphorylates PI3K products ($PI3,4,5P_3$) and inhibits AKT1 activity (3), alterations in this series of tumors. Three cases showed PTEN down-regulation. These data suggest that PI3K activation is primary reason to result in elevated AKT1 kinase levels in human ovarian cancer. The majority of the tumors with PI3K/AKT1 activation are late stages and high grades indicating that alterations of PI3K/AKT1 are associated with tumor progression rather than initiation.

II. Expression of constitutively activated AKT1 results in malignant phenotype in NIH 3T3 cells.

It has been demonstrated that wild type AKT1 was unable to transform NIH 3T3 cells (4). To determine whether activation of AKT1 in human tumors has biological implication, we have introduced HA-tagged constitutively active forms of Akt (Myr-Akt and Akt-E40K), wild type, and myristoylated kinase-inactive mutant (Myr-Akt-K179M) Akt into NIH 3T3 cells individually. After G418 selection, 5 stable clonal cell lines from each transfection were obtained. Cells transfected with constitutively active forms of Akt, but not myr-Akt-K179M and WT-Akt were morphologically transformed, grew in medium with low serum (0.1%), formed colonies in soft agar suspension, and were highly tumorigenic in nude mice. Tumors were observed 1-3 weeks after the injection of constitutively activated Akt-transfected cells in all mice (2). These data suggest that kinase activity of Akt/AKT1 is essential for oncogenic transformation in NIH 3T3 cells.

III. Inhibition of PI3K/Akt pathway induces apoptosis in human ovarian cancer cells.

To assess the PI3K/Akt as potential target for ovarian cancer intervention, 3 ovarian cancer cell lines with or without PI 3-kinase/AKT activation were treated with PI 3-kinase inhibitors, wortmannin or LY294002, or vehicle (DMSO) for 12 hours in a medium containing 1% fetal calf

serum. Those cell lines exhibiting elevated levels of PI3K/AKT activity underwent apoptosis after treatment with wortmannin or LY294002, whereas no apoptosis was detected in the cell line without PI 3K/AKT activation (5, see Appendix).

IV. Estrogen receptor (ER) α binds to and activates PI3K/Akt pathway which feedback regulates ERα transcriptional activity.

Although the role of ERα in ovarian oncogenesis is controversial, animal experimental data showed that estrogen significantly induces ovarian surface epithelial (OSE) cell proliferation (6). We have recently demonstrated that ERα physically interacts with p85α regulatory subunit of PI3K and induces PI3K/Akt pathway. AKT phosphorylated serine-167 of ERα *in vitro* and *in vivo* and stimulated its transcriptional activity (7, see Appendix). It has been shown that ERα can upregulate cyclin D1, c-myc, cathepsin D and TGFα that are known to stimulate cell proliferation. Therefore, regulation between PI3K/Akt pathway and ERα could contribute to control of OSE cell growth.

In addition, we collaborated with Dr. Patricia Kruk's group and identified that PI3K mediates stress-induced telomerase activity (8, see Appendix). Unfortunately, the grant number (DAMD17-00-1-0559) was forgotten putting in the paper.

KEY RESEARCH ACCOMPLISHMENTS:

1. Identification of frequent alterations of PI3K/AKT1 in human primary ovarian cancer which may associate with tumor progression.
2. Activation of AKT1 in human ovarian cancer has important biological implication in term of malignant transformation.
3. Inhibition of PI3K/AKT1 pathway induces apoptosis in human ovarian cancer cells.
4. Regulation between PI3K/Akt pathway and ERα could contribute to ovarian carcinogenesis.

REPORTABLE OUTCOMES:

1. Four manuscripts (see appendix).
2. One abstract in "17th Annual Meeting on Oncogene; Cancer Cell Signal Transduction".

CONCLUSIONS:

Our data demonstrated: 1) activation PI3K/AKT1 pathway is a common occurrence in human ovarian cancer; 2) activation of AKT1 results in malignant transformation; 3) inhibition PI3K/AKT1 pathway inhibits ovarian cancer cell growth and induces apoptosis and 4) PI3K/AKT1 regulates ERα and telomerase activity to control the OSE cell proliferation.

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APPENDICES:

1. Sun M, Wang, G, Paciga, JE, Feldman RI, Yuan Z, Ma X, Shelley SA, Jove R, Tsichlis PN, Nicosia SV, Cheng J.Q. AKT1/PKBα kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. *Am. J. Pathology* 159:431-437, 2001.
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Short Communication

AKT1/PKBα Kinase Is Frequently Elevated in Human Cancers and Its Constitutive Activation Is Required for Oncogenic Transformation in NIH3T3 Cells

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Extensive studies have demonstrated that the Akt/AKT1 pathway is essential for cell survival and inhibition of apoptosis; however, alterations of Akt/AKT1 in human primary tumors have not been well documented. In this report, significantly increased AKT1 kinase activity was detected in primary carcinomas of prostate (16 of 30), breast (19 of 50), and ovary (11 of 28). The results were confirmed by Western blot and immunohistochemical staining analyses with phospho-Ser473 Akt antibody. The majority of AKT1-activated tumors are high grade and stage III/IV (13 of 16 prostate, 15 of 19 breast, and 8 of 11 ovarian carcinomas). Previous studies showed that wild-type AKT1 was unable to transform NIH3T3 cells. To demonstrate the biological significance of AKT1 activation in human cancer, constitutively activated AKT1 (Myr-Akt) was introduced into NIH3T3 cells. Overexpression of Myr-Akt in the stably transfected cells resulted in malignant phenotype, as determined by growth in soft agar and tumor formation in nude mice. These data indicate that AKT1 kinase, which is frequently activated in human cancer, is a determinant in oncogenesis and a potential target for cancer intervention. ***(Am J Pathol 2001, 159:431–437)***

Akt, also known as protein kinase B, represents a subfamily of the serine/threonine protein kinases.[1–5] Akt/AKT1/PKBα signaling has been extensively studied throughout the last 6 years. It has been shown that Akt is activated by a variety of stimuli in a phosphoinositide-3-OH kinase (PI 3-kinase)-dependent manner.[6–9] Activation of Akt by growth factors depends on the integrity of the PH domain, which binds to the PI 3-kinase product, $PI(3,4,5)P_3$, and phosphorylation of Thr-308 and Ser-473 by PDK1 and PDK2/ILK, respectively. In addition, growth factor-induced Akt activation is also mediated by Ras, Src, and Gab1.[10–12] In numerous cell types, it has been shown that Akt induces survival and suppresses apoptosis induced by a variety of stimuli, including growth factor withdrawal and loss of cell adhesion. The mechanisms by which Akt promotes cell survival include phosphorylation of the pro-apoptotic proteins BAD, caspase-9, Forkhead transcription factors, and IκB kinase α, resulting in reduced binding of BAD to Bcl-X_L, inhibition of caspase-9 protease activity, Fas ligand gene transcription, and activation of the nuclear factor-κB cascade.[13–17] Akt has also been shown to inhibit the Raf-MEK-ERK pathway through phosphorylation of Raf-1 in myotubes and overcome constitutively activated MAPK-induced cell-cycle arrest in MCF7 cells.[18,19]

Although Akt/AKT1 is essential for cell survival and anti-apoptosis, alterations of Akt/AKT1 have not been consistently observed in any human malignancy. In fact, amplification of AKT1 has been reported in only a single gastric carcinoma.[20] In this communication, we describe frequent activation of AKT1 in human carcinomas of prostate, breast, and ovary. We also demonstrate the biological significance of AKT1 activation in human cancer by showing that constitutively activated, but not wild-type, Akt is highly oncogenic in NIH3T3 cells.

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M.S. and G.W. contributed equally to this work.

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Materials and Methods

Tumor Specimens, Cell Lines, Transfection, and Transformation Assay

All primary human cancer specimens were obtained from patients who underwent surgery at the H. Lee Moffitt Cancer Center and each sample contained at least 70% tumor cells as was confirmed by histological examination. The tissues were snap-frozen and stored at −70°C. Slides from each case were reviewed for grade and stage following the criteria of the American Joint Committee on Cancer, 1988 edition. NIH3T3 cells were cultured at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Transfection was performed with LipofectAMINE PLUS (Life Technologies, Inc., Rockville, MD). Stable clonal cell lines were established after G418 selection. Soft agar suspension and tumorigenesis assays were performed as previously described.[21]

Plasmids

Hemagglutin epitope (HA)-tagged wild-type, constitutively active (Myr-Akt and Akt-E40K), and dominant-negative (kinase-inactive mutant Myr-Akt-K179M) Akt were described previously.[22]

Immunoprecipitation and Western Blotting Analysis

The frozen tissue was lysed by a tissue tearor in a lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 137 mmol/L NaCl, 15% (v/v) glycerol, 1% Nonidet P-40, 2 mmol/L phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin, 2 mmol/L benzamidine, 20 mmol/L NaF, 10 mmol/L NaPPi, 1 mmol/L sodium vanadate, and 25 mmol/L β-glycerophosphate. An equal amount of protein was analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1) agarose beads at 4°C for 20 minutes. After removal of the beads by centrifugation, lysates were incubated with the indicated antibody in the presence of protein A-protein G (2:1) agarose beads for 2 hours at 4°C. The beads were washed once with 50 mmol/L Tris-HCl (pH 7.5), 0.5 mol/L LiCl, 0.5% Triton X-10, twice with phosphate-buffered saline (PBS), and once with 10 mmol/L Tris-HCl (pH 7.5), 10 mmol/L $MgCl_2$, 10 mmol/L $MnCl_2$, and 1 mmol/L dithiothreitol, all containing 20 mmol/L β-glycerophosphate and 0.1 mmol/L sodium vanadate. Immunoprecipitates were subjected to *in vitro* kinase assays or Western blotting analysis. Protein expression was determined by probing Western blots of immunoprecipitates with phospho-Ser473 Akt (New England Biolabs, Beverly, MA) or anti-AKT1 (Santa Cruz Biotechnology, Santa Cruz, CA) antibody. Detection of antigen-bound antibody was performed with the ECL Western blotting analysis system (Amersham, Arlington Heights, IL).

Immunohistochemistry

Formalin-fixed paraffin-embedded sections were deparaffinized in xylene and rehydrated through graded alcohol to distilled water. The sections were subjected to antigen retrieval by boiling in a microwave for 30 minutes in 0.01 mol/L sodium citrate buffer (pH 6.0) and then exposed to 3% hydrogen peroxide diluted in water for 20 minutes at room temperature to block endogenous peroxidase activity. Sections were incubated in a blocking solution (PBS containing blocking serum) for 20 minutes followed by 20 minutes with an avidin/biotin blocking solution (Vector Laboratories, Burlingame, CA). The primary antibody to phospho-S473 Akt (Upstate Biotechnology, Lake Placid, NY) or PTEN (Upstate Biotechnology) was applied and incubated overnight at 4°C. After incubation, the slides were treated with biotinylated secondary antibody, washed, and treated with streptavidin and biotinylated horseradish peroxidase according to the manufacturer's instruction (Vector Laboratories, Burlingame, CA). After washing, the signal was visualized by diaminobenzidine tetrahydrochloride. A negative control reaction with no primary antibody was always performed alongside the reaction-containing sample.

In Vitro *Protein Kinase Assay*

In vitro Akt kinase assays were performed as previously described.[11] Briefly, the reaction was performed in the presence of 10 μCi of [γ-^{32}P]ATP (New England Nuclear, Boston, MA) and 3 μmol/L of cold ATP in 30 μl of buffer containing 20 mmol/L Hepes (pH 7.4), 10 mmol/L $MgCl_2$, 10 mmol/L $MnCl_2$, and 1 mmol/L dithiothreitol. Histone H_2B was used as the exogenous substrate. After incubation at room temperature for 30 minutes, the reactions were stopped by adding protein-loading buffer, and the products were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Each experiment was repeated three times. The relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

PI 3-Kinase Assay

Anti-p85 (Santa Cruz) antibody was used to immunoprecipitate p110 catalytic subunits of PI 3-kinase from the tumor lysate. The immunoprecipitates were washed once with cold PBS, twice with 0.5 mol/L LiCl, 0.1 mol/L Tris (pH 7.4), and finally with 10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid. The presence of PI 3-kinase activity in immunoprecipitates was determined by incubating the beads with reaction buffer containing 10 mmol/L HEPES (pH 7.4), 10 mmol/L $MgCl_2$, 50 μmol/L ATP, 20 μCi [γ-^{32}P]ATP, and 10 μg L-α-phosphatidylinositol-4,5-bis phosphate (PI-4,5-P_2; Biomol, Plymouth Meeting, PA) or L-α-phosphatidylinositol-4-phosphate (Sigma Chemical Co., St. Louis, MO) for 20 minutes at 25°C. The reactions were stopped by adding 100 μl of 1 mol/L HCl. Phospholipids were

extracted with 200 μl of $CHCl_3CH_3$/MeOH. Phosphorylated products were separated by thin-layer chromatography as previously described.[23] The conversion of PI-4,5-P_2 to PI-3,4,5-P_3 and PI-4-P_1 to PI-3,4-P_2 was determined by autoradiography and quantitated by using a PhosphorImager.

Results

Frequent AKT1 Activation in Human Cancer

Despite the fact that the PI 3-kinase/Akt/AKT1 pathway is essential for cell survival and anti-apoptosis, consistent alterations of AKT1 in human primary tumors have not been well documented. We have previously examined AKT1 and AKT2 alterations at the DNA and/or mRNA levels in more than 100 cancer cell lines (including NCI 60 cancer cell lines screen) and more than 300 primary tumors from various organs. Amplification and/or overexpression of AKT2 were observed in 15 to 25% of ovarian and pancreatic tumors examined,[2,24,25] whereas no AKT1 alteration at the DNA or mRNA level was detected (J. Q. Cheng and J.R. Testa, unpublished data). The essential role of AKT1 kinase in cell survival prompted us to examine if AKT1 kinase activity is elevated in human cancer. We first examined whether anti-AKT1 antibody (D-17, Santa Cruz) specifically recognizes AKT1. HEK293 cells were transiently transfected with HA-AKT1, HA-AKT2, or HA-AKT3, lysed, and immunoprecipitated with monoclonal anti-HA antibody. The immunoprecipitates were separated and detected with AKT1 antibody. Figure 1A showed that anti-AKT1 antibody only reacted with HA-AKT1. We next immunoprecipitated AKT1 from lysates prepared from frozen tumors of prostate, breast, and ovary with anti-AKT1 antibody. The AKT1 immunoprecipitates were subjected to *in vitro* kinase assays. Significantly increased AKT1 kinase activity was observed in 16 of 30 prostate adenocarcinomas, 19 of 42 ductal breast cancers, and 11 of 23 ovarian serous adenocarcinomas. No elevated AKT1 activity was detected in eight lobular breast carcinomas, three endometrioid, and two borderline ovarian cancers examined, implying that alteration of AKT1 kinase level primarily involves ductal breast and serous ovarian carcinomas. Moreover, we observed that the majority of AKT1 activated-tumors are high grade and stage III/IV (Table 1), suggesting that activation of AKT1 plays an important role in tumor progression rather than initiation.

Table 1. Frequencies of AKT1 Activation and Tumor Stage and Grade

		Kinase activity		
	n	Normal	High	*P* value
Stage				
Prostate cancer				
T1–T2	12	9	3	0.013§
T3–T4	18	5	13	
Breast cancer				
I–II	19	15	4	0.038§
III–IV	31	16	15	
Ovarian cancer				
I–II	9	7	2	0.155
III–IV	19	10	9	
Grade				
Prostate cancer				
<7*	16	10	6	0.055
>7	14	4	10	
Breast cancer				
1–2†	16	14	2	0.009§
3‡	34	17	17	
Ovarian cancer				
1–2†	11	8	3	0.186
3‡	17	9	8	

*Gleason score.
†Well (1) or moderately (2) differentiated.
‡Poorly differentiated.
§$P < 0.05$.

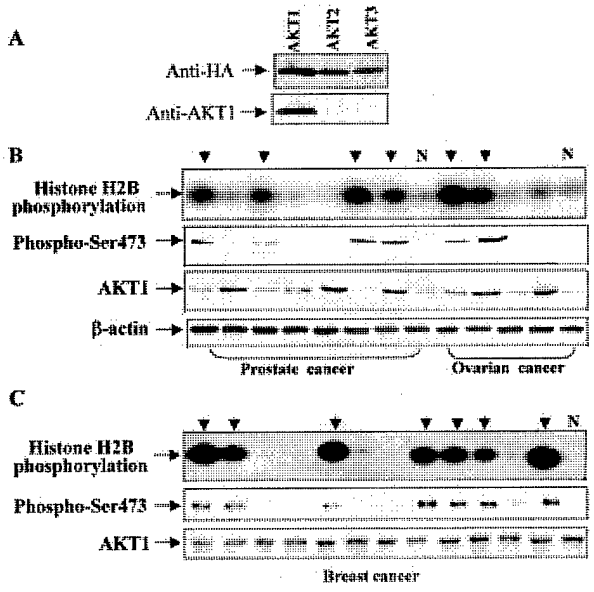

Figure 1. Activation of AKT1 in human cancers. **A:** Western blot. HEK293 cells were transiently transfected with HA-AKT1, HA-AKT2, or HA-AKT3 expression construct, lysed, immunoprecipitated with anti-HA antibody, and detected with anti-HA (**top**) or anti-AKT1 antibody (**bottom**). The top panels of **B** and **C** are *in vitro* kinase assays of AKT1 immunoprecipitates from frozen tumor specimens of prostate and ovary (**B**), and breast (**C**). AKT1 kinase levels were highly elevated in cases indicated by **arrows**. Normal tissue lysates (N) from prostate, ovary, and breast were used as controls. The second panels of **B** and **C** are Western blot analyses of AKT1 immunoprecipitates probed with anti-phospho-Ser473 Akt antibody. The cases exhibiting elevated AKT1 kinase activity displayed phosphorylation bands. The third and fourth panels of **B** and bottom panel of **C** are Western blot analyses with anti-AKT1 and anti-β-actin antibodies.

It has been shown that phosphorylation of threonine-308 and serine-473 is required for activation of AKT1 and that phospho-Ser473 Akt antibody recognizes only the phosphorylated/active form of Akt.[9] To confirm the results obtained from *in vitro* kinase assays, we performed Western blotting analysis with this antibody. To avoid the possibility of cross-reaction of the phospho-Ser473 antibody with other isoforms of Akt/PKB family, the tissue lysates were first immunoprecipitated with the specific anti-AKT1 antibody. The AKT1 immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with anti-phospho-Ser473 antibody. Phosphorylated AKT1 was detected only in the

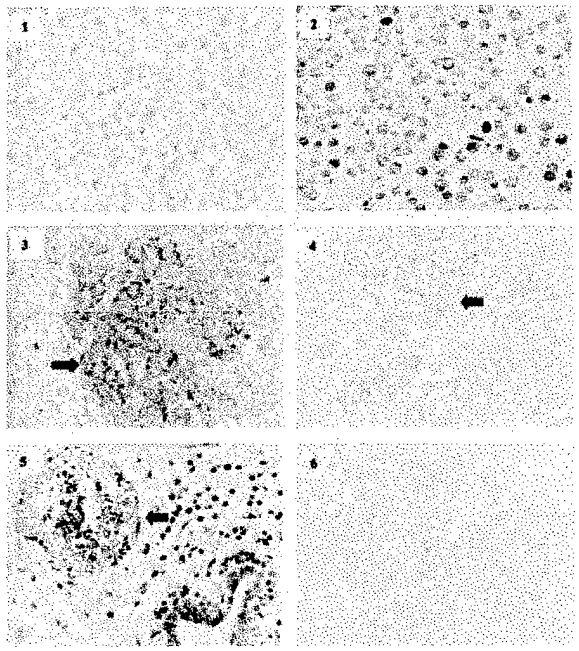

Figure 2. Phospho-AKT1 was detected in tumor cells and located only in cytoplasm. Immunochemical staining of the paraffin sections prepared from serum-starved (**1**), EGF-stimulated MCF7 cells (**2**), and adenocarcinomas of ovary (**3**), prostate (**4**), and breast (**5** and **6**) with phospho-S473 Akt antibody. Strong staining was observed in EGF-stimulated MCF7 cells and ovarian, breast, and prostate tumor cells, indicated by **black arrows**. No immunoreaction was detected in unstimulated MCF7 cells (**1**) normal prostate gland and ductal epithelial cells (**4** and **5**, **white arrow**), and the breast tumor without elevated AKT1 activity (**6**).

tumors with elevated AKT1 kinase activity (second panels of Figure 1, B and C).

To further demonstrate AKT1 activation and determine whether activated AKT1 is derived from tumor cells or stromal tissues in the tumor specimens with elevated AKT1 activity identified by *in vitro* kinase assay, immunohistochemical staining of tumor paraffin sections was performed with phospho-Ser473 Akt antibody. We first demonstrated that the phospho-Ser473 Akt antibody is capable of recognizing phosphorylated AKT1 by immunostaining paraffin sections prepared from serum-starved and serum-starved/EGF-stimulated MCF7 cells (Figure 2, panels 1 and 2). Phosphorylation status of AKT1 in these cells was confirmed by Western blot analysis with phospho-S473 Akt antibody (data not shown). The tumor paraffin sections from the 16 prostate, 19 breast, and 11 ovarian tumor specimens with elevated AKT1 kinase activity strongly immunoreacted with phospho-Ser473 Akt antibody (Figure 2, panels 3 to 5), whereas no immunostaining was observed in normal tissues and the tumor samples without increased AKT1 activity (Figure 2, panel 6). Interestingly, phosphorylated AKT1 was located in the tumor cell membrane and cytoplasm but not the nucleus, which is in conflict with the previously reported observation that activated Akt could translocate to the nucleus in ectopically Akt-overexpressing cells.[26,27] Our data also showed that overexpression of GFP-tagged AKT1 translocates to the nucleus in NIH3T3 cells after IGF-1 stimulation (data not shown),

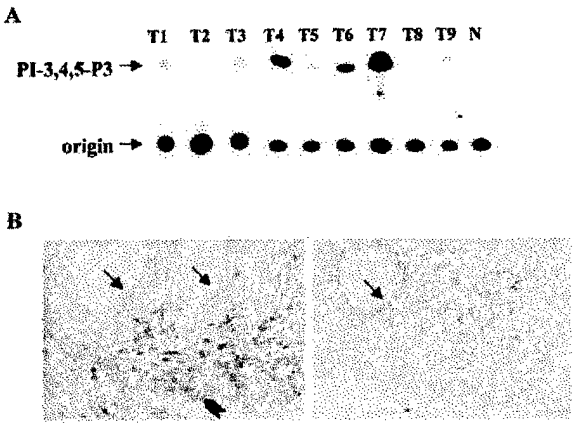

Figure 3. Activation of PI 3-kinase or down-regulation of PTEN in human tumors. **A:** *In vitro* PI 3-kinase assay of the anti-p85 immunoprecipitates from nine tumor specimens that exhibit elevated AKT1 kinase activity. Elevated levels of PI 3-kinase activity were detected in cases 4, 6, and 7. **B:** Immunostaining of paraffin sections of prostate adenocarcinomas with anti-PTEN (**left**) and anti-phospho-Ser473 Akt (**right**) antibodies. PTEN is negative in tumor cells (**black arrows**), but positive in hyperplastic glands (**arrowhead**). However, phosphorylation of Akt was only detected in tumor cells (**black arrow**).

indicating that activated Akt in the primary tumor cells could have a different subcellular localization from the cells overexpressing exogenous Akt.

Multiple Mechanisms Resulted in AKT1 Activation

Because AKT1 kinase activity is regulated positively by PI 3-kinase and negatively by *PTEN/MMAC1*,[28] we examined PI 3-kinase activity and PTEN expression in the tumors exhibiting AKT1 activation (Figure 3). Elevated PI 3-kinase activity was observed in 7 of the 19 breast and 5 of the 11 ovarian carcinomas, but in none of the prostate tumors that exhibit AKT1 activation. Immunohistochemical staining revealed no PTEN expression in 10 of 16 prostate and 2 of 11 ovarian cancer specimens with elevated AKT1 activity, whereas all breast carcinomas that showed AKT1 activation expressed PTEN. Absence of PTEN is well correlated with positive staining of phosphorylated AKT1 on the tumor tissue sections (Figure 3 and data not shown). In addition, we performed single-strand conformational polymorphism/sequencing analyses in AKT1-activated tumor specimens that have neither PI 3-kinase nor PTEN alteration. No AKT1 mutation was detected, implying that there are other mechanisms leading to AKT1 activation in the specimens without alterations of either PI 3-kinase or PTEN, which needs further investigation.

Constitutively Active Forms of Akt/AKT1 Are Highly Oncogenic in NIH3T3 Cells

We and others previously demonstrated that overexpression of wild-type of Akt (WT-Akt) is unable to transform NIH3T3 cells.[21,29] To determine whether activation of AKT1 in human tumors has biological implication, we

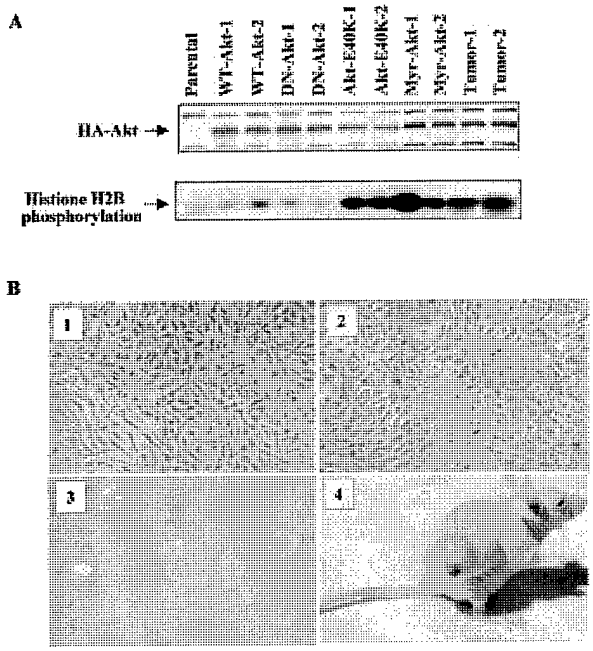

Figure 4. Constitutively activated AKT1 transforms NIH3T3 cells. **A:** Western blot (**top**) and *in vitro* kinase assay (**bottom**) analyses of Akt expression and kinase activity in stably transfected clones and a tumor sample from nude mice. For Western blot analysis, the lysates were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to membrane, and detected with anti-HA antibody. For assay of kinase activity, immunoprecipitation was performed with anti-HA antibody and the HA-Akt immunoprecipitates were subjected to *in vitro* kinase assay using histone H2B as substrate. **B:** The morphology of constitutively active Akt-transfected NIH3T3 cells (**2**) is more rounded and larger than WT-Akt-transfected cells (**1**). Constitutively activated Akt-transfected cells grew on soft agar (**3**) and formed tumors in nude mice (**4**).

have introduced HA-tagged constitutively active forms of Akt (Myr-Akt and Akt-E40K), wild-type, and myristoylated kinase-inactive mutant (Myr-Akt-K179M) Akt into NIH3T3 cells individually. After G418 selection, five stable clonal cell lines from each transfection were obtained. Western blot and *in vitro* kinase analyses revealed that all of the clonal cell lines express Akt protein (Figure 4A). High levels of kinase activity were detected in constitutively active Akt (Myr-Akt and Akt-E40K)-transfected clonal cell lines. Figure 4A shows Akt expression and kinase activity in two clonal cell lines from each transfection. Cells transfected with constitutively active forms of Akt, but not Myr-Akt-K179M and WT-Akt were morphologically transformed, grew in medium with low serum (0.1%), formed colonies in soft agar suspension, and were highly tumorigenic in nude mice (Figure 4B). Tumors were observed 1 to 3 weeks after the injection of constitutively activated Akt-transfected cells in all mice, except clone 2 of Akt-E40K cells (Figure 4 and Table 2). Although vector alone and WT-Akt-transfected NIH3T3 cells developed tumors in 1 of 25 and 2 of 25 mice, respectively, all of these tumors were detected after 38 days (Table 2). In addition, high levels of Myr-AKT1 protein and kinase activity were observed in dissected tumors from Myr-Akt nude mice but not in the tumors from vector or WT-Akt mice (Figure 4A). These data suggest that kinase activity of Akt/AKT1 is essential for oncogenic transformation in NIH3T3 cells.

Table 2. Tumorigenicity of Akt and Control Transfectant NIH3T3 Cell Lines

Clonal cell lines	Soft agar growth*	Tumorigenicity in nude mice†	Latency (days)
Akt-E40K 1	+++	5/5	14–18
Akt-E40K 2	++	4/5	17–22
Akt-E40K 3	+++	5/5	15–21
Akt-E40K 4	+++	5/5	13–18
Akt-E40K 5	+++	5/5	13–18
Myr-Akt 1	+++	5/5	11–14
Myr-Akt 2	+++	5/5	8–10
Myr-Akt 3	+++	5/5	10–11
Myr-Akt 4	+++	5/5	7–10
Myr-Akt 5	+++	5/5	8–11
Myr-Akt-K179M (1–5)	–	0/25	
Vector control (1–5)	–	1/25	40
WT-Akt (1–5)	–	2/25	38–42

*Number of colony/60 mm plate: 1–10, +; 11–30, ++; >30, +++.
†The number of injected cells: 1×10^6/mouse.

Discussion

Activation of oncogenic signaling proteins, such as Stat3, mitogen-activated protein kinase, and p110α, has been demonstrated in a number of different tumors.[30–32] Constitutively activated Stat3 and PI 3-kinase are able to induce malignant transformation.[33,34] Recent studies showed that among the most critical tumor-cell survival pathways are those mediated by the Akt/AKT1 kinase.[6–9] In this report, we demonstrate frequently elevated AKT1 kinase activity and phosphorylation of AKT1 in human carcinomas of breast, prostate, and ovary. Moreover, we have also shown that constitutively activated, but not wild-type, AKT1 is highly tumorigenic in NIH3T3 cells. Furthermore, the majority of AKT1-activated tumors are high grade and stage III/IV. These results indicate that activation of AKT1 is a common occurrence in human cancer, especially in more advanced tumors.

We previously demonstrated that overexpression of wild-type AKT2, but not Akt/AKT1, in NIH3T3 cells resulted in malignant transformation.[21] Ahmed and colleagues[29] also showed that Akt is not tumorigenic when overexpressed in the nontumorigenic rat T-cell lymphoma cell line 5675. In contrast, *v-akt*-expressing 5675 cells were highly tumorigenic. Because v-*akt* arose by an in-frame fusion of the viral *Gag* and *Akt*, the oncogenic difference between v-akt and wild-type Akt/AKT1 may be because of myristoylation of the amino-terminus of v-akt.[29,35] Several lines of evidence show that attachment of a membrane-targeting sequence (myristoylation/palmitoylation) to the amino-terminus of AKT1/Akt is sufficient to induce its maximal activation and that the PH domain of Akt is required for its membrane translocation and activation.[9] We demonstrated, in this study, that overexpression of constitutively active forms of *Akt* (Myr-Akt and Akt-E40K) in NIH3T3 cell leads to oncogenic transformation, which supports the results obtained from chicken embryo fibroblasts and Rat1 cells.[36,37] Taken collectively, these data suggest that the kinase activity of Akt contributes to the control of cell malignant transformation and that elevated AKT1 kinase activity plays an

important role in development and/or progression of a subset of human cancers.

Previous studies have demonstrated that all of the tumor-associated *PTEN* mutants that have been biochemically characterized result in activation of AKT1.[28] Recently, elevated PI 3-kinase activity has been observed in human ovarian cancer.[32,38] As discussed above, Ras, Src, and Gab1 mediate growth factor signals to activate the PI 3-kinase/Akt pathway.[10–12] Therefore, activation of AKT1 in human cancer could result from Ras mutation, overexpression/active mutation of growth factor receptor(s), AKT1 mutation, and Src activation. In the present report, we showed that the majority of cases with AKT1 activation had either PTEN down-regulation or PI 3-kinase activation, dependent on the tumor type. Activation of PI 3-kinase was frequently detected in breast and ovarian, but not prostate, carcinomas, whereas the absence of PTEN protein was observed in some prostate and ovarian carcinomas. However, missense mutations of *PTEN*, although uncommon, cannot be ruled out by immunohistochemistry, because they result in formation of full-length PTEN proteins that may be immunostained by anti-PTEN antibody. Nevertheless, no AKT1 mutation was found in the tumors examined, indicating that elevated AKT1 activity in human cancer results from alterations of upstream regulators of AKT1.

Amplification and/or overexpression of AKT2, but not AKT1, have been detected in a subset of human ovarian, pancreatic, and breast cancers,[2,24,25] suggesting that AKT2 may play a more important role in human malignancy. In the present study, frequent activation of AKT1 kinase in human cancers and malignant transformation resulting from expression of constitutively activated AKT1 provide the first evidence that AKT1 could have a role similar to that of AKT2 in human cancer. Comparison of the AKT and AKT2 protein and/or kinase levels in the same tumor will provide valuable information to better understanding of the importance of AKT1 and AKT2 in human malignancy. Expression of AKT1 and AKT2 protein and alterations of AKT2 at the kinase level in these series of specimens are currently under investigation.

Subcellular localization of activated AKT1 is controversial.[9] Early studies on the subcellular localization of Akt/AKT1 revealed that, whereas c-akt is primarily cytosolic, v-akt is distributed equally between the cell membrane, the cytoplasm, and the nucleus in NIH3T3 cells.[29] Recent studies showed that nuclear translocation of AKT1 and AKT2 in HEK293 and HeLa cells follows in short succession the insulin-induced translocation of AKT1 and AKT2 proteins to the cell membrane.[26,27] However, all of these studies were performed in AKT1- or AKT2-transfected cells. In this report, we demonstrated that activated AKT1 in human primary tumors is distributed only in the plasma membrane and the cytosol, suggesting that activated endogenous AKT1 may not translocate to nucleus. In addition, we noted that immunoreaction to the phospho-Ser-473 antibody in tumor specimens is less strong, which could be because of either weak epitope of single phosphopeptide or epitope masking in paraffin section.

In summary, the data presented in this report showed that AKT1 kinase is frequently activated in human prostate, breast, and ovarian carcinomas. Elevated AKT1 kinase is an essential requirement for its oncogenic activity. These results provide the basis for understanding how the Akt pathway contributes to human oncogenesis. Further studies are required to determine the clinicopathological significance of AKT1 activation and to examine if overexpression of constitutively activated AKT1 develops prostate, breast, and ovarian tumors in transgenic mouse models using tissue-specific promoters.

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Advances in Brief

Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, Activated in Breast Cancer, Regulates and Is Induced by Estrogen Receptor α (ERα) via Interaction between ERα and PI3K[1]

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Abstract

We have shown previously that the AKT2 pathway is essential for cell survival and important in malignant transformation. In this study, we demonstrate elevated kinase levels of AKT2 and phosphatidylinositol-3-OH kinase (PI3K) in 32 of 80 primary breast carcinomas. The majority of the cases with the activation are estrogen receptor α (ERα) positive, which prompted us to examine whether AKT2 regulates ERα activity. We found that constitutively activated AKT2 or AKT2 activated by epidermal growth factor or insulin-like growth factor-1 promotes the transcriptional activity of ERα. This effect occurred in the absence or presence of estrogen. Activated AKT2 phosphorylates ERα *in vitro* and *in vivo*, but it does not phosphorylate a mutant ERα in which ser-167 was replaced by Ala. The PI3K inhibitor, wortmannin, abolishes both the phosphorylation and transcriptional activity of ERα induced by AKT2. However, AKT2-induced ERα activity was not inhibited by tamoxifen but was completely abolished by ICI 164,384, implicating that AKT2-activated ERα contributes to tamoxifen resistance. Moreover, we found that ERα binds to the p85α regulatory subunit of PI3K in the absence or presence of estradiol in epithelial cells and subsequently activates PI3K/AKT2, suggesting ERα regulation of PI3K/AKT2 through a nontranscriptional and ligand-independent mechanism. These data indicate that regulation between the ERα and PI3K/AKT2 pathway (ERα-PI3K/AKT2-ERα) may play an important role in pathogenesis of human breast cancer and could contribute to ligand-independent breast cancer cell growth.

Introduction

Breast cancer development and tumor growth are strongly associated with estrogens. The binding of an estrogen molecule to the ERα[3] induces a cascade of events, including the release of accessory proteins (*e.g.*, the heat-shock proteins), increased nuclear retention, DNA binding, and the transcription of estrogen-responsive genes, such as cyclin D1, c-myc, cathepsin D, and transforming growth factor-α that are known to stimulate mammary cell proliferation (1). ERα is a member of a superfamily of nuclear receptors that act as transcription factors when bound to specific lipophilic hormones. In common with other steroid hormone receptors, the ERα has a NH_2-terminal domain with a hormone-independent transcriptional activation function (AF-1), a central DNA-binding domain, and a COOH-terminal ligand-binding domain with a hormone-dependent transcriptional activation function (AF-2; Refs. 2, 3). In addition to its ligand, estradiol, the ERα is also activated by several nonsteroidal growth factors including EGF and IGF1 through their cell membrane receptors and cytoplasmic signaling pathways such as MAPK signal transduction pathway (3, 4). Because of the role of ERα in promoting the growth and progression of breast cancers, considerable efforts are devoted to development of reagents to functionally inactivate ERα, so as to inhibit ERα-mediated gene expression and cell proliferation. Antiestrogens such as tamoxifen and ICI 164,384 antagonize the effects of estrogens by competing with estrogen for binding to ERα. Tamoxifen or its derivative 4-hydroxytamoxifen inhibits transcriptional activation by AF-2 but not AF-1 (5). ICI 164,384, on other hand, is a complete antagonist that blocks transcriptional activation by both AF-1 and AF-2 (6). However, approximately one-third of ERα-positive breast cancers fail to respond to antiestrogen treatment, which is thought to result from growth factor-induced ERα activity through activation of protein kinases resulting in phosphorylation of ERα (7).

It has been well documented that phosphorylation of ERα is essential for the activation of ERα after stimulation with its ligand and nonsteroidal growth factors (EGF and IGF1). The phosphorylation of ERα is observed at both serine and tyrosine residues. The serine residues are the predominant modified amino acids present in ERα, and four of these (Ser-104, Ser-106, Ser-118, and Ser-167) are clustered in the NH_2 terminus within the AF-1 region. Phosphorylation of ERα at Ser-118 is mediated by the Ras/MAPK pathway; therefore, activation of the MAPK pathway enables ligand-independent transactivation of ERα (4). There is evidence showing that Ser-167 is phosphorylated by several protein kinases, including casein kinase II and $pp90^{rsk1}$, which is important for DNA binding and transcriptional activation (8, 9). Phosphorylation of ERα on tyrosine 537, which is required for ERα dimerization and transactivation, by Src family tyrosine kinases *in vitro* has also been demonstrated. Moreover, protein kinase A has been shown to phosphorylate ERα at Ser-236 and regulate its dimerization (10).

In addition, recent studies (11) demonstrated that plasma membrane ERα plays a crucial role in transducing cellular signals. It has been convincingly shown that ERα activates G-protein-coupled receptor leading to the modulation of downstream pathways that have discrete cellular actions including membrane K^+ and Ca^{2+} channel activation and induction of protein kinase C and protein kinase A kinase activity (11). A recent study (12) demonstrated that estrogen activates p38 MAPK, resulting in the activation MAPK-protein kinase-2 and subsequent phosphorylation of heat shock protein 27. ERα has been also shown to interact with IGF1R and induce IGF1R and extracellular signal-regulated kinase activation (13).

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[3] The abbreviations used are: ERα, estrogen receptor α; PI3K, phosphatidylinositol-3-OH kinase; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; HA, hemagglutinin; EGF, epidermal growth factor; IGF1, insulin-like growth factor-1; GST, glutathione *S*-transferase; HEK, human embryonic kidney.

Akt, also called protein kinase B, has been identified as a direct target of PI3K (14). All of the three members, Akt/AKT1/PKBα, AKT2/PKBβ, and AKT3/PKBγ, of this family are activated by growth factors in a PI3K-dependent manner (14–16). Numerous studies (17) showed that the Akt pathway is critical for cell survival by phosphorylation of a number of downstream proteins including BAD, caspase-9, Forkhead transcription factors, IKKα, Raf, and p21-activated protein kinase. Among Akt family members, AKT2 has been shown to be predominantly involved in human malignancies such as ovarian and pancreatic cancers (18–20). In this study, we demonstrate frequent activation of AKT2 and PI3K in human breast cancer. AKT2 phosphorylates ERα at Ser-167 and activates ERα-mediated transcription in a PI3K-dependent manner. ERα binds to the p85α subunit of PI3K in epithelial cells and activates the PI3K/AKT2 pathway in an estrogen-independent manner.

Materials and Methods

Tumor Specimens, Cell Lines, and Transfection. All of the 80 primary human breast cancer specimens were obtained from patients who underwent surgery at H. Lee Moffitt Cancer Center, and each sample contained at least 70% tumor cells, as was confirmed by microscopic examination. The tissues were snap-frozen and stored at −70°C. ERα-negative epithelial HEK293 and COS7 cells and ERα-positive MCF7 and BG-1 cells were cultured at 37°C and 5% CO_2 in DMEM supplemented with 10% FCS. The cells were seeded in 60-mm Petri dishes at a density of 8×10^5 cells/dish. Transfections were performed by calcium phosphate DNA precipitation or Lipofectamine Plus (Life Technologies, Inc.).

Immunoprecipitation and Western Blotting Analysis. The cells and frozen tumor tissues were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15% (volume for volume) glycerol, 1% NP40, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin, 2 mM benzamidine, 20 mM NaF, 10 mM $NaPP_i$, 1 mM sodium vanadate, and 25 mM β-glycerol phosphate. Lysates were centrifuged at 12,000 × *g* for 15 min at 4°C before immunoprecipitation or Western blotting. The protein concentration in each tissue lysate was measured, and an equal amount of protein was analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1) agarose beads at 4°C for 20 min. After removal of the beads by centrifugation, lysates were incubated with anti-AKT2 (Upstate Biotechnology) antibody in the presence of 30 μl of protein A-protein G (2:1) agarose beads for 2 h at 4°C. The beads were washed once with 50 mM Tris-HCl (pH 7.5)-0.5 M LiCl-0.5% Triton X-10, twice with PBS, and once with 10 mM Tris-HCl (pH 7.5)-10 mM $MgCl_2$-10 mM $MnCl_2$-1 mM DTT, all containing 20 mM β-glycerol phosphate and 0.1 mM sodium vanadate. Immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Protein phosphorylation and expression were determined by probing Western blots of immunoprecipitates with anti-phospho-Akt-Ser473 (Cell Signaling) or anti-AKT2 antibody. Detection of antigen-bound antibody was carried out with the enhanced chemiluminescence Western Blotting Analysis System (Amersham).

***In Vitro* Protein Kinase Assay.** Akt kinase assay was performed as described previously (15). Briefly, the reaction was carried out in the presence of 10 μCi of [γ-^{32}P]ATP (NEN) and 3 μM cold ATP in 30 μl of buffer containing 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM DTT using histone H2B as substrate. After incubation at room temperature for 30 min, the reaction was stopped by adding protein-loading buffer, and the mixture was separated in SDS-PAGE gels. Each experiment was repeated three times. The relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a Phosphorimager (Molecular Dynamics).

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections were subjected to antigen retrieval by boiling in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven after dewaxing and rehydration. The Vectastain ABC Kit for sheep IgG (Vector Laboratories) was used to immunostain the tissue sections with phospho-S473 Akt antibody (catalogue number 06-801-MN; Upstate Biotechnology). Endogenous peroxidase and biotin were blocked, and sections were incubated 1 h at room temperature with a 1:250 dilution of antibody to phospho-S473 Akt. The remainder of the staining procedure was performed according to the manufacturer's instructions using diaminobenzidine tetrahydrochloride as the chromogen and hematoxylin for counterstaining. Primary antibody was replaced with an equal concentration of nonimmune sheep IgG on negative control sections.

PI3K Assay. PI3K was immunoprecipitated from the tumor tissue lysates with pan-p85 antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed once with cold PBS, twice with 0.5 M LiCl, 0.1 M Tris (pH 7.4), and finally with 10 mM Tris, 100 mM NaCl, 1 mM EDTA. The presence of PI3K activity in immunoprecipitates was determined by incubating the beads with reaction buffer containing 10 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 50 μM ATP, 20 μCi [γ-^{32}P]ATP, and 10 μg of L-a-phosphatidylinositol-4,5-bis phosphate (PI-4,5-P_2; BIOMOL) for 20 min at 25°C. The reactions were stopped by adding 100 μl of 1 M HCl. Phospholipids were extracted with 200 μl of $CHCl_3$/methanol. Phosphorylated products were separated by TLC as described previously (21). The conversion of PI-4,5-P_2 to PI-3,4,5-P_3 was determined by autoradiography and quantitated by using a Phosphorimager. Average readings of the kinase activity 3-fold higher than that in normal ovarian tissue was considered as elevated PI3K activity.

Expression Constructs and GST Fusion Protein. HA epitope-tagged constitutively active, wild-type, and dominant-negative AKT2 were prepared as described previously (21). The p110α and p85α of PI3K expression constructs were gifts from Dr. Julian Downward (Imperial Cancer Research Fund, London, United Kingdom). The mammalian expression construct of ERα-S167A was kindly provided by Dr. Benita S. Katzenellenbogen (University of Illinois, Urbana, IL). The GST-ERα and GST-ERα-S167A were created by PCR and inserted into pcDNA3 and pEGX-4T (Pharmacia) vectors, respectively. GST-ERα fusion proteins were purified as described previously (21).

***In Vivo* [^{32}P]P_i Cell Labeling.** Transfected COS7 and nontransfected MCF7 cells were labeled with [^{32}P]P_i (0.5 mCi/ml) in MEM without phosphate, serum, and phenol red for 4 h and lysed. ERα was immunoprecipitated with monoclonal anti-ERα or anti-myc antibody. The immunoprecipitates were separated on SDS-PAGE and transferred to membranes. Phosphorylated ERα was detected by autoradiography and quantitated by using Molecular Dynamics Phosphorimager with ImageQuant software.

Reporter Assay. HEK293 and MCF7 cells (8×10^5) were seeded in a 60-mm plate. The cells were cotransfected with the luciferase reporter plasmid (2ERE-MpG12), wild-type, constitutively active, or dominant-negative AKT2 and ERα, as well as pCMV-βgal plasmid as an internal control. The amount of DNA in each transfection was kept constant by the addition of empty pcDNA3 vector. Luciferase and β-galactosidase activities were determined 48 h after transfection according to the manufacturer's procedure (Promega). Luciferase activity was corrected for transfection efficiency by using the control β-galactosidase activity. All of the experiments were performed in triplicate from independent cell cultures.

Results and Discussion

Frequent Activation of AKT2 in Breast Carcinoma. We have demonstrated previously (15, 20) that AKT2, like AKT1, is activated by a number of mitogenic growth factors in a PI3K-dependent manner and that AKT2 kinase activity is frequently elevated in human ovarian tumors. To examine whether AKT2 is activated in human primary breast cancer, we performed *in vitro* kinase assays in 80 human breast carcinoma specimens, including 58 ductal infiltrating adenocarcinomas, 16 lobular carcinomas, and six mixed tumors. Lysates from tumor specimens were incubated with anti-AKT2 antibody, which specifically reacts with AKT2 (20). The immunoprecipitates were subjected to *in vitro* kinase assay using histone H2B as substrate. The results revealed an elevated level of AKT2 kinase in 32 of specimens (40%), including 29 cases with ductal infiltrating carcinoma, two lobular, and one mixed tumor (Fig. 1*A*). To further demonstrate AKT2 activation in breast cancer, we performed Western blot analyses of tumor lysates with phospho-Ser-473 antibody, a phosphorylation site that is critical for activation of three isoforms of Akt (17). To avoid the cross-reaction, the tumor lysates were incubated with anti-AKT2 antibody. The AKT2 immunoprecipitates were separated by SDS-PAGE and probed with phospho-Ser473 antibody. Phosphorylated AKT2 was detected only in breast tumors with elevated AKT2 kinase

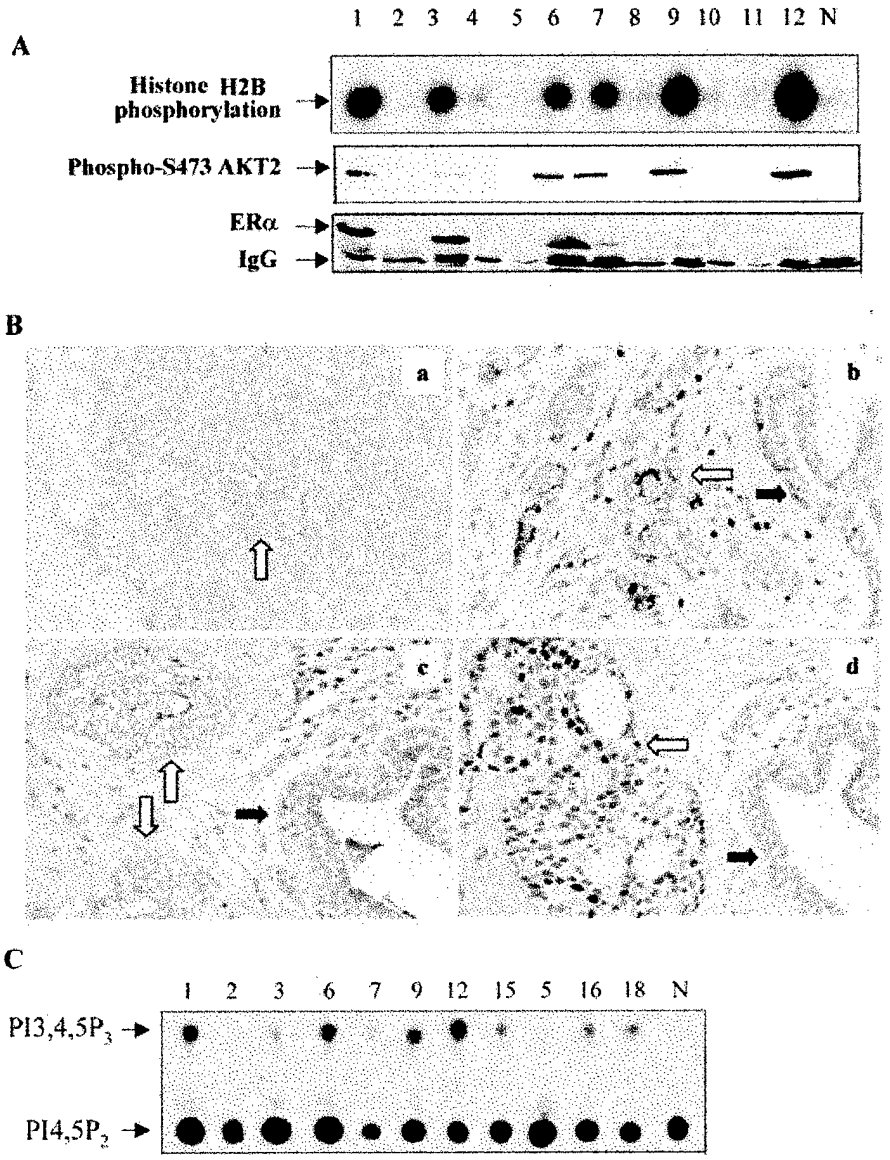

Fig. 1. Activation of AKT2 in human primary breast cancers. *A (top panel), in vitro* kinase assays of immunoprecipitated AKT2 from representative frozen breast tumor specimens. Normal mammary tissue (*N*) was used as a control. *Bottom panels*, Western blot analyses of AKT2 and ERα immunoprecipitates with anti-phospho-Ser473 Akt and anti-ERα antibodies, respectively. *B*, immunochemical staining of the paraffin sections prepared from primary breast adenocarcinomas with anti-phospho-S473 Akt (*a–c*) and anti-ERα (*d*) antibodies. Strong staining with both antibodies was observed in tumor cells (*white arrows*), whereas weak immunoreaction was detected in stromal tissue and adjacent ductal epithelium (*black arrows*). Photomicrographs *c* and *d* are the same specimen but different sections. *C, In vitro* PI3K assay of anti-p85 immunoprecipitates from 11 tumor and one normal specimen. The specimen numbers correspond to the same tumors shown in *A*.

activity (Fig. 1*A*). Because stromal tissues account for approximately 20–30% of the tumor specimens used in this study, we examined whether the activation of AKT2 is derived from the tumor cells or the stromal tissues by immunostaining paraffin sections with a phospho-Ser473 Akt antibody. Positive staining of tumor cells was detected in all of the 32 cases with AKT2 activation, whereas no staining was observed in normal ductal epithelial cells (Fig. 1*B*). These data suggest that activation of AKT2 is a common occurrence in human breast cancer.

Because AKT2 is a downstream target of PI3K, which is activated in colon and ovarian carcinoma (20, 22, 23), we next examined the PI3K activity in breast tumors by *in vitro* PI3K assay. Because of the fact that all of the tumors with elevated PI3K activity result in activation of Akt (20, 22–24), immunoprecipitation with a pan-p85 antibody was performed in 58 breast tumor specimens, including 32 with AKT2 activation and, as control, 26 without AKT2 activation. The ability to convert PI-4,5-P_2 to PI-3,4,5-P_3 was determined. Elevated PI3K activity was detected in all of the 32 specimens that exhibited AKT2 activation. No PI3K activation was observed in 26 specimens without AKT2 activation (Fig. 1*C*), indicating that activation of AKT2 in breast cancer predominantly results from PI3K activation. Moreover, Western blot and immunohistochemistry analyses with anti-ERα antibody revealed that 88% of the cases (28 of 32) with PI3K/AKT2 activation showed strong ERα positive (Fig. 1, *A* and *B*), whereas only 54% of the cases (14 of 26) without PI3K/AKT2 activation exhibited positive ERα, suggesting that activated PI3K/AKT2 could be involved in the regulation of ERα activity in breast cancer cells. In addition, the majority of cases with AKT2 activation are late stage (23 of 32 at stages III and IV) and poorly differentiated tumors (19 of 32), indicating that PI3K/AKT2 activation in breast cancer may be associated with tumor progression rather than initiation.

AKT2 Activates ERα-mediated Transcription in a Ligand-independent Manner. Previous studies (1, 25) have shown that MAPK is activated in breast cancer and contributes to estrogen-independent breast tumor cell growth by direct phosphorylation of ERα. Moreover, several other signal molecules, including protein kinase A, casein kinase II, $pp90^{rsk1}$, and MEKK1/p38, have been

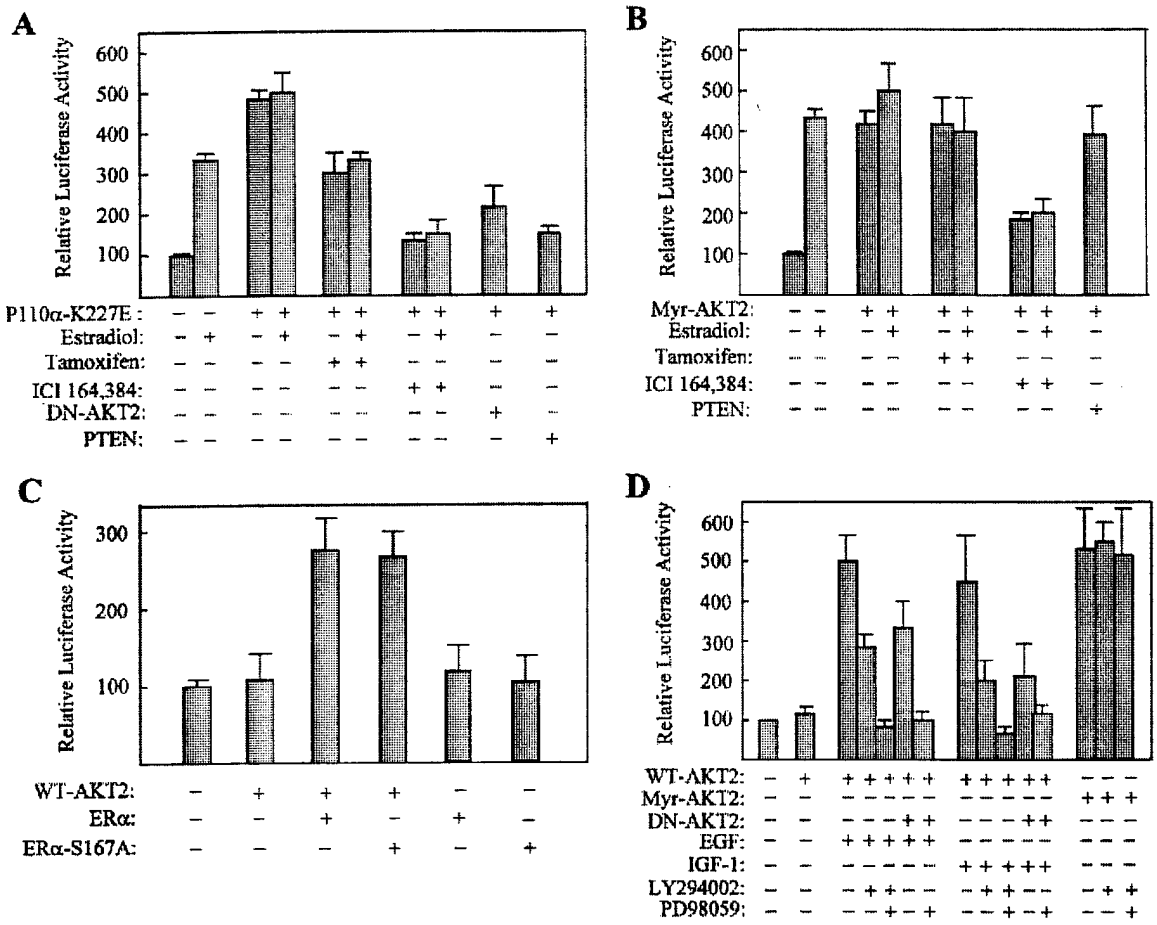

Fig. 2. AKT2 and PI3K activate ERα transcriptional activity. *A–D*, reporter assays: MCF-7 cells were transfected with ERE2-TK-LUC reporter, β-galactosidase, and indicated expression constructs. After 36 h of transfection, the cells were serum-starved overnight and treated with indicated agents. Luciferase activity was normalized to β-galactosidase activity.

shown to activate ERα-mediated transcription, possibly resulting in hormone-independent tumor cell growth (1, 8–10, 26). Because AKT2 and PI3K are frequently activated in breast cancer and the majority of cases with AKT2 activation are ERα positive, we investigated whether AKT2 and PI3K regulate ERα-mediated transcription. ERα-positive MCF7 breast cancer cells were transiently transfected with a reporter construct containing a luciferase gene regulated by two estrogen response elements (ERE2-TK-LUC) and a plasmid expressing β-galactosidase that allows the luciferase data to be normalized for transfection efficiency. In addition, the cells were transfected with expression constructs for constitutively activated p110α (p110α-K227E) subunit of PI3K, wild-type, constitutively activated, and dominant-negative AKT2 or vector alone. As shown in Fig. 2, p110α-K227E or myr-AKT2 increased ERE2-TK-LUC activity 3–4-fold in the absence of estradiol. Constitutively activated p110α-induced reporter activity was attenuated by dominant-negative mutant AKT2 (Fig. 2*A*). Tamoxifen (4-hydroxytamoxifen), an antiestrogen reagent that inhibits transcriptional activation by AF2 but not through AF1 (5), abolished estradiol-enhanced transcription but had no effects on p110α-K227E and myr-AKT2-stimulated ERα activity (Fig. 2, *A* and *B*), suggesting that PI3K/AKT2-increased ERα transcriptional activity is regulated by phosphorylation of ERα within the AF1 region and could be involved in tamoxifen resistance.

ICI 164,384, which causes rapid degradation of ERα (6, 27), completely blocked PI3K- and AKT2-induced reporter activity. PTEN, a tumor suppressor encoding a lipid phosphatase that negatively regulates PI3K, inhibited constitutively active p110-induced ERα-mediated transcription but had no effect on constitutively activated AKT2-stimulated ERα activity (Fig. 2*B*).

Moreover, we have observed that exogenous expression of ERα in ERα-positive MCF7 cells increased wild-type AKT2-induced ERE2-TK-LUC activity 2–3-fold as compared with cells transfected with wild-type AKT2 alone (Fig. 2*C*), implying that ERα might activate AKT2 kinase and subsequently enhance its own transcriptional activity (see below). Taken collectively, these data indicate that PI3K/AKT2-activated ERα-mediated transcription is estrogen-independent and that the frequently elevated level of PI3K/AKT2 kinase in primary breast cancer could relate the refractoriness of hormone therapy.

AKT2 Mediates Growth Factor-induced ERα Transcriptional Activity. A very recent study (28) showed that Akt1 mediates the estrogenic functions of EGF and IGF1. Next, we examined the possible role of AKT2 in growth factor-induced ERα activation. ERα-positive MCF7 cells were transfected with ERE2-TK-LUC and dominant-negative, wild-type, or constitutively activated AKT2 or vector alone and were treated with or without either 100 ng/ml EGF or 50 ng/ml IGF1 (Fig. 2*D*). Treatment with the growth factors resulted in an approximately 4.5-fold increase in ERα-mediated transcriptional activity. The EGF- and IGF1-induced reporter activity was partially abrogated by dominant-negative AKT2 or PI3K inhibitor LY294002 and completely blocked by the combination of PI3K and MAPK inhibitors (LY294002 and PD98059). However, the combined inhibitors had no effect on constitutively activated AKT2-induced reporter

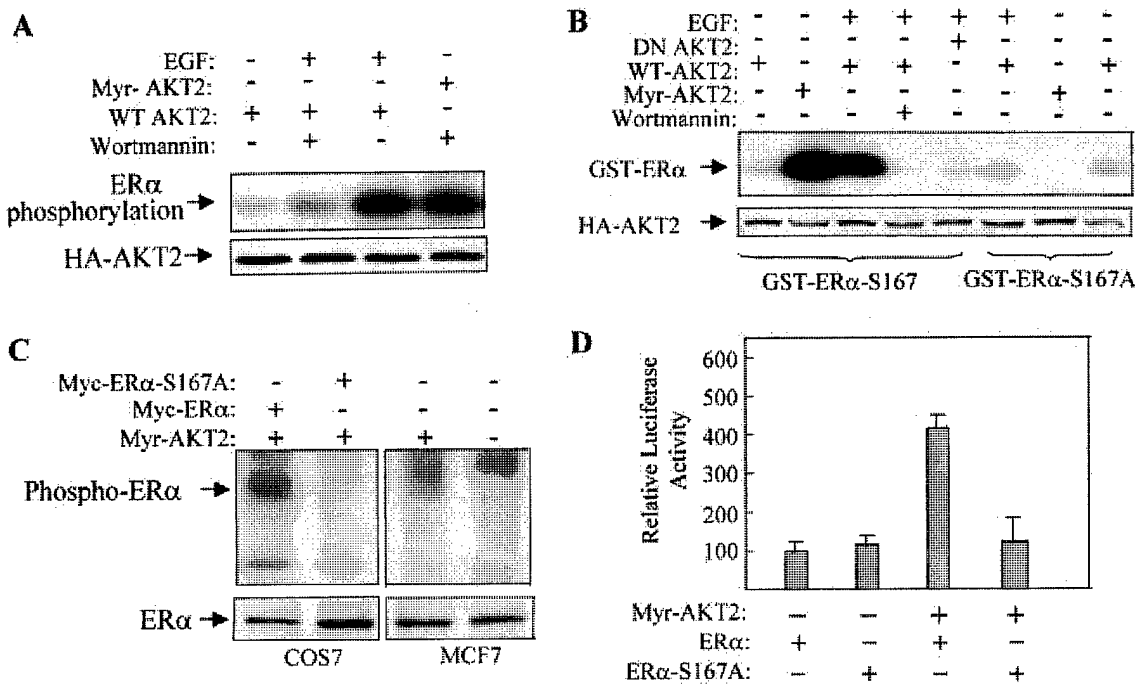

Fig. 3. AKT2 phosphorylates ERα on serine-167 *in vitro* and *in vivo*. *In vitro* AKT2 kinase assay of the immunoprecipitates from HEK293 cells transfected with indicated expression constructs. Full length of human recombinant ERα (*A*), GST-ERα-S167, and GST-ERα-S167A (*B*) were used as substrates. *C*, COS7 and MCF7 cells were transfected with indicated plasmids and incubated with $[^{32}P]P_i$ for 4 h. Immunoprecipitates were prepared with anti-myc (*left*) or anti-ERα (*right*) antibody and separated by SDS-PAGE. After transfer, the membrane was exposed to a film (*top*) and detected with anti-ERα antibody (*bottom*). *D*, AKT2 phosphorylation of serine-167 is essential for AKT2-induced ERα transcriptional activity. Luciferase reporter assay of HEK293 cells transfected with ERE2-TK-LUC, wild-type ERα, or ERα-S167A, β-galactosidase, and myr-AKT2.

activity. These results suggest that the "steroid-independent activation" of ERα by growth factors is mediated by the PI3K/AKT2 pathway, in addition to MAPK, PKA, casein kinase II, and $pp90^{rsk1}$.

AKT2 Phosphorylated Serine-167 of ERα *in Vitro* and *in Vivo*. Phosphorylation of ERα has been shown to be an important mechanism by which ERα activity is regulated. ERα is hyperphosphorylated on multiple sites in response to hormone binding and growth factor stimulation (1–4). Transcriptional activation by growth factors has been shown to require AF-1 but not AF-2 (1, 8–10). There is evidence to suggest that EGF and IGF-1 induce MAPK and $pp90^{rsk1}$/casine kinase II activity leading to phosphorylation of serine-118 and serine-167, respectively, in AF-1 region (4, 5, 8–10). To examine whether AKT2 phosphorylates ERα *in vitro*, HEK293 cells were transfected with HA-tagged wild-type and constitutively activated AKT2, and immunoprecipitation was prepared with anti-HA antibody. *In vitro* AKT2 kinase assays, using full length of human recombinant ERα as substrate, revealed that constitutively activated AKT2 and EGF-induced AKT2 strongly phosphorylated hERα. The ERα phosphorylation that was induced by EGF-stimulated AKT2 was abrogated by wortmannin (Fig. 3*A*).

To determine whether AKT2 phosphorylates ERα *in vivo*, MCF7 cells were transfected with constitutively activated AKT2 or pcDNA3 vector alone and labeled with $[^{32}P]P_i$. The cell lysates were incubated with anti-ERα antibody, and the immunoprecipitates were separated on SDS-PAGE. ERα was highly phosphorylated in constitutively activated AKT2-transfected cells but in the cells transfected with vector alone (Fig. 3*C*). These data indicate that AKT2 phosphorylated ERα both *in vitro* and *in vivo*.

Martin *et al.* (28) recently demonstrated that EGF- and IGF1-induced Akt1 potentiates the AF-1 function of ERα, possibly through the phosphorylation of serine residues. There are four serine residues (Ser-104, Ser-106, Ser-118, and Ser-167) in the AF-1 region of the receptor that are predominantly phosphorylated in response to estrogen and growth factor stimulation (1–4). We examined the ERα protein sequence and found that serine-167 (^{162}**RERLAS**^{167}) is a putative AKT2 phosphorylation site. Constructs expressing GST-fused wild-type and mutant (S167A) AF-1 region were created. *In vitro* kinase assays revealed that myr-AKT2 and EGF-stimulated AKT2 strongly phosphorylated GST-ERα-S167 but not GST-ERα-S167A mutant (Fig. 3*B*). The EGF-induced AKT2 phosphorylation of ERα is blocked by wortmannin. To examine whether AKT2 phosphorylates serine-167 *in vivo*, COS7 cells were transfected with myc-tagged wild-type and mutant (S167A) human ERα expression constructs together with constitutively activated AKT2. After 36 h of transfection, the cells were incubated with $[^{32}P]P_i$ and immunoprecipitated with anti-myc antibody. As demonstrated in Fig. 3*C*, constitutively active AKT2 phosphorylated wild-type ERα but not the ERα-S167A mutant *in vivo*, suggesting that serine-167 of ERα is a physiological substrate for AKT2.

Previous studies (29) showed that serine-167 is important for ERα transcriptional activity. To further examine whether AKT2-activated ERα transcriptional activity depends upon phosphorylation of serine-167, reporter assays were carried out in HEK293 cells transfected with ERE2-TK-LUC, constitutively activated AKT2, and ERα-S167A or wild-type ERα. Fig. 3*D* shows that ERα-S167A had no ability to mediate constitutively activated AKT2-induced ERE2-TK-LUC reporter activity, indicating that AKT2 regulates ERα-mediated transcription through phosphorylation of serine-167.

ERα Binds To and Activates PI3K/AKT2 in Epithelial Cells via a Ligand-independent Mechanism. Recent studies (30, 31) demonstrated that ERα binds to the p85α regulatory subunit of PI3K after estradiol treatment, leading to the activation of PI3K/Akt and endo-

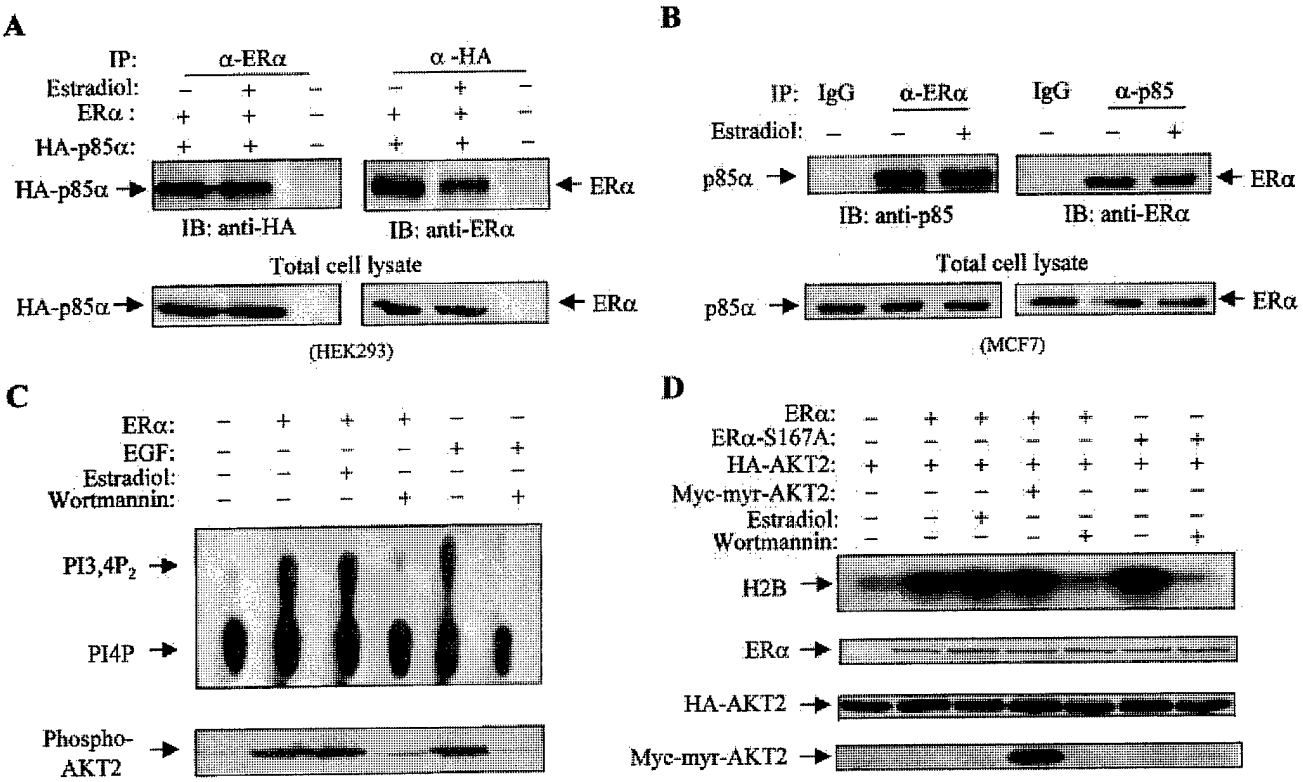

Fig. 4. ERα interacts with p85α and activates the PI3K/AKT2 pathway in human epithelial cells. Coimmunoprecipitation of ERα and p85α in (*A*) HEK293 cells cotransfected with HA-p85α/ERα and in (*B*) nontransfected MCF7 cells. *Top*, coimmunoprecipitation; *bottom*, Western blot of total cell lysates. *C*, *in vitro* PI3K assay (*top*) of HEK293 cells transfected and treated with indicated plasmid and agents. *Bottom*, Western blotting analysis of AKT2 immunoprecipitates with phospho-S473 Akt antibody. *D*, *in vitro* kinase assay (*top*) of HA-AKT2 immunoprecipitates prepared from HEK293 cells transfected with indicated expression constructs, using histone H2B as a substrate. *Panels 2–4*, Western blots of transfected HEK293 cell lysates detected with anti-ERα, anti-HA, or anti-myc antibody.

thelial nitric oxide synthase in endothelial cells. In the absence of estradiol, ERα failed to bind and activate PI3K, indicating that ERα-associated PI3K in endothelial cells is estrogen-dependent (30). Next, we examined whether ERα binds to and activates PI3K/AKT2 in epithelial cells. ERα/HA-p85α-transfected HEK293 and nontransfected ERα-positive MCF7 cells were immunoprecipitated with anti-ERα and detected with anti-HA or anti-p85α antibody or *vice versa*. As shown in Fig. 4*A* and *B*, ERα constitutively associated with p85α, and this interaction was unaffected by estradiol treatment. In addition, *in vitro* PI3K assays revealed that expression of ERα in HEK 293 cells significantly induced PI3K activity in the absence or presence of estradiol (Fig. 4*C*). These data suggest that ERα binding to and activating PI3K is ligand-independent in epithelial cells.

Next, we examined whether ERα activates AKT2 and whether this activation is dependent on AKT2 phosphorylation. ERα-negative HEK293 cells were transfected with ERα or ERα-S167A, together with HA-AKT2. *In vitro* AKT2 kinase assays revealed that ERα significantly activates AKT2 in the absence of estradiol. Additional estradiol treatment did not further enhance ERα-induced AKT2 activation. The PI3K inhibitor, wortmannin, completely abolished the activation. Interestingly, ERα-S167A activated AKT2 at a similar level to that of wild-type ERα. Coexpression of myc-tagged constitutively active AKT2 (Myc-myr-AKT2) and ERα had the same effect on wild-type AKT2 activation as that of expression of ERα alone (Fig. 4*D*). These results indicate that activation of AKT2 by ERα is through PI3K and independent of ERα phosphorylation by PI3K/AKT2.

In summary, we demonstrate in this study that AKT2 and PI3K are frequently activated in primary human breast carcinoma. The PI3K/AKT2 pathway regulates ERα transcriptional activity by phosphorylation of serine-167 *in vitro* and *in vivo*, and ERα activates PI3K/AKT2 kinase by binding to p85α in a ligand-independent manner in epithelial cells. This study suggests that the PI3K/AKT2 pathway may play a pivotal role in estrogen-independent breast cancer cell growth and tamoxifen-resistance; therefore, it could represent an important therapeutic target in human breast cancer.

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Ultraviolet Irradiation- and Dimethyl Sulfoxide-Induced Telomerase Activity in Ovarian Epithelial Cell Lines

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Information about telomerase regulation is incomplete, especially since various studies suggest complexity in telomerase regulation. Given the important association between telomerase and cancer, it is imperative to design and develop a model system in which telomerase activity can be regulated and studied. We employed ultraviolet (UV) radiation or dimethyl sulfoxide (DMSO) to transiently induce telomerase activity in a telomerase-positive cell line and, most importantly, in a telomerase-negative cell line. UV- or DMSO-induced telomerase activity was associated with increased hTRT, but not hTR, mRNA transcription in the telomerase-negative cells. However, no changes in hTRT or hTR mRNA transcription were noted with UV- or DMSO-induced telomerase activity in the telomerase-positive cells. Inhibition of protein synthesis or the phosphotidyl inositol 3-kinase (PI3K) pathway suppressed telomerase induction and/or activity in all cell lines examined, suggesting telomerase activity was dependent on protein synthesis and PI3K-mediated phosphorylation. Furthermore, enhanced telomerase activity was limited to UV and DMSO, since a variety of chemotherapeutic agents failed to induce telomerase activity. Therefore, our data provide a useful culture model system to study telomerase regulation in telomerase-negative and -positive cell lines and from which to obtain information about telomerase as a target for cancer intervention. © 2001 Academic Press

Key Words: **telomerase; ovarian cancer; UV irradiation; hTRT; PI 3-kinase; dimethyl sulfoxide; wortmannin; LY294002; hTR; cycloheximide.**

INTRODUCTION

Telomerase is a ribonucleoprotein that synthesizes telomeric repeats *de novo.* Telomerase is composed of three subunits: an RNA component (hTR), an additional protein component (TLP1), and a catalytic component (hTRT). While little information is available about the TLP1 component, the RNA component has been shown to be constitutively expressed in most cells [1] and serves as the template for the telomerase reverse transcriptase to lay down telomeric repeats. Studies suggest that hTRT is the rate-limiting component essential for telomerase activity and that it is transcriptionally regulated independently of hTR [2–5]. In addition, telomerase regulation appears to be complex, requiring the coordinated involvement of numerous proteins, including telomeric binding proteins TRF1 and TRF2, as well as tankyrase [6–9]. While reports correlating telomerase activity with cell cycle regulation are contradictory [10–14], several studies report abrogation of telomerase activity with differentiating agents, including dimethyl sulfoxide (DMSO) [15, 16], deoxynucleoside analogues [17], tea catechins [18], and the reverse transcriptase inhibitor AZT [19]. Molecular mechanisms of telomerase regulation include methylation of the hTRT gene promoter [20] and *c*-Myc-driven transcription of hTRT [21–23], resulting in increased telomerase activity. A recent report of hTRT protein phosphorylation by Akt kinase [24] implies a possible role for the phosphotidyl inositol 3-kinase (PI3K) signal transduction pathway in the regulation of telomerase activity.

Telomerase is present in more than 90% of all tumors, including ovarian cancer, which has the highest mortality rate among gynecological cancers (5-year survival is 37%). Furthermore, telomerase activity has been correlated with tumor stage and aggressiveness in tumors of breast, head/neck, lung, gastrointestines, pancreas, liver, prostate, kidney/bladder, brain, skin, and ovary as well as leukemic and lymphomatous tumors [25]. Since activated Akt is frequently detected in breast, prostate, and ovarian carcinomas [26], and since Akt, a downstream target of PI3K, appears to play a role in telomerase regulation, we sought to develop a controlled culture model system to study telomerase regulation in ovarian cancer. We employed ultraviolet (UV) or DMSO to transiently elevate endogenous telomerase activity in a telomerase-positive cell line and, more importantly, to transiently induce *de novo*

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telomerase activity in a telomerase-negative cell line. UV- or DMSO-induced telomerase activity was associated with increased hTRT mRNA levels only in previously telomerase-negative cell lines. Induced telomerase activity was not related to hTR mRNA levels, but appeared to require protein synthesis in all cell lines examined. We demonstrated an increase in PI3K activity following UV treatment [27–33]. We also showed, for the first time, that DMSO can also increase PI3K activity. Consequently, inhibition of PI3K suppressed telomerase activity, further supporting a role for PI3K-mediated regulation of telomerase activity. Therefore, not only have we developed a model for telomerase induction in a telomerase-positive cell line but, more importantly, we also were able to induce telomerase activity in a previously telomerase-negative cell line. This model system provides a useful tool for the study of telomerase regulation in order to evaluate telomerase as a potential target for cancer intervention.

METHODS AND MATERIALS

Cell culture. Two ovarian carcinoma cell lines, SW626 and CaOV3 (obtained from ATCC, Rockville, MD) were used. Although the origin of the SW626 cell line has been questioned [34], this cell line has been described as an ovarian carcinoma cell line. Four nontumorigenic, SV40 large-T-antigen-transfected ovarian surface epithelium cell lines (IOSE) derived from normal ovarian surface epithelium [35], FHIOSE 135, FHIOSE 118, NFHIOSE 80, and NFHIOSE 144, were also used in the present study. All IOSE cell lines were previously determined to be telomerase negative [36]. Cells were maintained in Medium 199/MDCB 105 (1:1) (Sigma, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 10 μg/ml gentamicin (GIBCO BRL, Grand Island, NY) in a humidified 5% CO_2/95% air atmosphere.

Ultraviolet irradiation. Exponentially growing cultures of IOSE and SW626 cells were rinsed with Dulbecco's phosphate-buffered saline (DPBS), drained, and irradiated with UV radiation at 2–40 J/m^2 using a 254-nm germicidal lamp. Altering the exposure time controlled for the total dose of radiation. Following irradiation, fresh medium was added and cells were incubated until their collection at various time intervals. Control samples were rinsed with DPBS and drained, and fresh medium was added.

Treatment with chemotherapeutic and chemical agents. To examine the effect of chemotherapeutic agents on telomerase activity, cells were treated with carboplatinum (CB), etoposide (ET), or cisplatinum (CP). CB, ET, and CP were dissolved in fresh, serum-free medium. Doses used were CB, 1, 10, and 25 μM; ET, 10, 50, and 100 μM; and CP, 25 and 100 μM. In order to determine the effects of a differentiating agent on telomerase activity, cells were treated with DMSO. Final concentrations of DMSO ranged from 0.05 to 1.0% in serum-free medium. For treatment, cells were rinsed with DPBS and the medium containing an agent was added. Controls consisted of cells that received only serum-free medium. Following 2 h of treatment, control and experimental cells were rinsed with DPBS and fresh medium containing 5% FBS was added. Cells were then harvested at various time intervals following treatment.

Treatment with cycloheximide. To determine if new protein synthesis was required for induction of telomerase activity, SW626 and FHIOSE 118 cells were treated with cycloheximide (CHX). CHX was dissolved directly into Medium 199/MDCB 105 (1:1) for a final concentration of 40 μM. For UV-treated cells, CHX-containing medium was added to exponentially growing cells immediately following UV treatment until the time of collection. For DMSO-treated cells, DMSO was added to the CHX-containing medium at the previously described concentrations. When the DMSO treatment period was completed, cells were rinsed twice with DPBS and fresh CHX-containing medium was added until the time of collection.

Telomerase assay. To quantitatively detect changes in telomerase levels, all cells were assayed for telomerase activity using the telomerase polymerase chain reaction–enzyme-linked immunosorbent assay (PCR–ELISA) (Roche Molecular Biochemicals, Indianapolis, IN), as described previously [36] and according to the manufacturer's instructions. This assay has been demonstrated to be as sensitive as the radioactive, telomere repeat amplification protocol (TRAP) assay [37–44]. Briefly, cells were washed with DPBS, trypsinized, counted, and centrifuged at 500*g* for 5 min. Pellets were washed twice in DPBS, then resuspended in 200 μl of lysis buffer and kept on ice for 30 min, after which time the lysates were centrifuged at 100,000*g* for 60 min at 4°C. Lysates were then assayed using the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA) for the determination of protein concentration following detergent solubilization, according to the manufacturer's instructions. In order to perform the telomerase PCR–ELISA within a linear range, IOSE and SW626 cell extracts equivalent to 2 μg of protein were used. Due to its high level of telomerase activity, the volume of cell extract used for the CaOV3 cell line, which served as a positive control, was equivalent to 1 μg of protein. Following PCR–ELISA, telomerase activity was detected using a Dynex-MRX plate reader (Dynex Technologies, Chantilly, VA) and recorded as absorbance units. These values were expressed as a fold increase above control levels, with the control value used as the denominator for the determination of fold increase for the treated samples. Telomerase activity is shown ±SE if the experiments were repeated in triplicate.

Treatment with inhibitors of PI3K. SW626 and FHIOSE 118 cells were treated with wortmannin (WM) and/or LY294002 (LY), potent and specific inhibitors of PI3K [45–48], to determine if the PI3K signal transduction pathway was involved in the regulation of telomerase. For these experiments, all cells were initially serum-starved for 12 h. Following serum starvation, cells were treated with PI3K inhibitors, 100 nM WM or 10 μM LY, in serum-free medium for 12 h while parallel cultures were maintained in serum-free medium. When WM was used, the medium was replaced with fresh serum-free, WM-containing medium every 90 min due to the short half-life of WM. Cells were then UV-irradiated at 30 J/m^2 or treated with 0.5% DMSO as described earlier, or remained untreated. Triplicate samples of SW626 and FHIOSE 118 cells were collected at 8 and 24 h, respectively, and assayed for telomerase activity. In addition, RNA was isolated from SW626 and FHIOSE 118 cells at 4, 8, or 16 h following treatment to determine the levels of hTRT and hTR mRNA by reverse transcription polymerase chain reaction (RT–PCR).

PI 3-kinase assay. In order to determine if PI3K was involved in transduction of the signal that regulates telomerase activity, a PI 3-kinase assay was performed [49, 50]. SW626 and FHIOSE 118 cells were treated with UV or DMSO, as described above. Thirty minutes following treatment, cells were rinsed once with cold DPBS and lysed with a protein lysis buffer (137 mM NaCl, 20 mM Tris, pH 8.0, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μM aprotonin, 2 μM leupeptin, 20 mM NaF, 10 mM NaPPi, 1 mM Na_3VO_4, 25 mM β-GP, 1 mM DTT, 2 mM benzamidine). The lysates were first assayed for protein concentration, as described previously, to ensure equal amounts of protein per sample. The lysates were then briefly vortexed and centrifuged for 5 min at 12,000*g* and 4°C. To preclear the protein samples, supernatants were transferred to fresh Eppendorf tubes containing 25 μl protein A:protein G Sepharose beads (2:1) and 3 μl rabbit serum and then incubated at 4°C for 20 min. Samples were then centrifuged for 1 min at 6000*g* and 4°C. Supernatants were transferred to fresh Eppendorf tubes containing 4 μg of anti-p110α (Upstate Biotechnology, Lake Placid, NY) and incubated for 1 h at 4°C. Thirty microliters

of Protein A Sepharose beads was added to each sample, followed by a second 1 h-incubation at 4°C. Samples were then centrifuged for 1 min at 5800*g* and 4°C. Supernatants were discarded and beads were washed once with 1 ml of buffer 1 (DPBS and 1 mM PMSF, 1 mM DTT, 0.1 mM Na_3OV_4), twice with 1 ml of buffer 2 (0.5 M LiCl, 0.1 M Tris, pH 7.4, 1 mM PMSF, 1 mM DTT, 0.1 mM Na_3OV_4), and once with 1 ml of buffer 3 (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, 0.1 mM Na_3OV_4). Between washes, samples were centrifuged for 1 min at 5800*g* and 4°C. The PI-4,5-P_2 substrate (Sigma) was dissolved in chloroform (1 mg/ml). Ten microliters of substrate solution was then vacuum dried for 3 min, redissolved with 25 μl of 20 mM Hepes at pH 7.4, and sonicated in a water bath for 20 min. This substrate solution was then added to the washed beads and incubated on ice for 20 min. Twenty-five microliters of the final reaction mixture (5 mM $MgCl_2$, 50 μM ATP, 20 μCi [γ-^{32}P]ATP) was then added to each sample and incubated at room temperature for 20 min. The reaction was terminated with 100 μl 1 M HCL. The phospholipids were then extracted with 200 μl of $CHCL_3$:MeOH (1:1), and the organic phase was washed once with 100 μl of MeOH:1M HCl (1:1). The organic phase was then vacuum dried, redissolved in 20 μl of $CHCl_3$:MeOH (1:1), and spotted onto a silica gel plate (pretreated with 1% potassium oxalate and baked at 50°C for 1 h). Phosphorylated lipids were separated with $CHCl_3$: MeOH:4 M NH_4OH (9:7:2), visualized with a Storm Phosphorimager, and analyzed using ImageQuant software. The results are described as fold increase.

RT-PCR. To examine the contribution of transcriptional control in telomerase regulation, RT–PCR studies were performed, with each experiment repeated a minimum of three separate times. Total RNA was collected using TRizol reagent (GIBCO BRL). One microgram total RNA, oligo(dT), and reverse transcriptase were used to generate single-strand cDNA for each sample, as previously described [51]. To insure there was no DNA contamination, each sample for reverse transcription was prepared in duplicate, with the duplicate preparation lacking reverse transcriptase [52]. The cDNA samples were amplified using the Perkin–Elmer (Palo Alto, CA) GeneAmp kit. The hTRT primers used were hTRT-S (CGGAAGAGTGTCTGGAGCCAA) and hTRT-AS (GGATGAAGCGGAGTCTGGA) oligonucleotides (Sigma Genosys, The Woodlands, TX) with β-actin primers actin-S (CAGGTCATCACCATTGGCAATGAGC) and actin-AS (GATGTCCACGTCACACTTCATGA) for an internal control. PCR was performed for 33 cycles of 95°C for 20 s, 68°C for 40 s, and 72°C for 30 s. β-Actin primers were added at cycle 16. For hTR, the primers used were hTR-46S (CTAACCCTAACTGAGAAGGGCGTAG) and hTR-148AS (GAAGGCGGCAGGCCGAGGCTTTTCC). PCR was performed for 28 cycles of 95°C for 20 s, 68°C for 40 s, and 72°C for 30 s. β-Actin primers were added at cycle 13 [51]. The amplified products were then separated by electrophoresis on a 9% polyacrylamide gel, stained with 1× SyberGreen (FMC Bioproducts, Rockland, ME), and analyzed with the Kodak EDAS 120 Digital Analysis System. Net hTRT and hTR mRNA intensities from treated samples were normalized to their corresponding β-actin mRNA levels and were expressed as a percentage of the control samples that were similarly normalized to their corresponding β-actin mRNA levels. For graphical representation, SW626 controls were set at 100%, while 118 controls (telomerase negative) were set at 0%.

Statistical analysis. Samples for telomerase PCR–ELISA were run in triplicate and the data subjected to Student *t* test analysis for determination of statistical significance for telomerase induction or suppression between treated and untreated samples. The standard error was shown only if the experiment was repeated in triplicate.

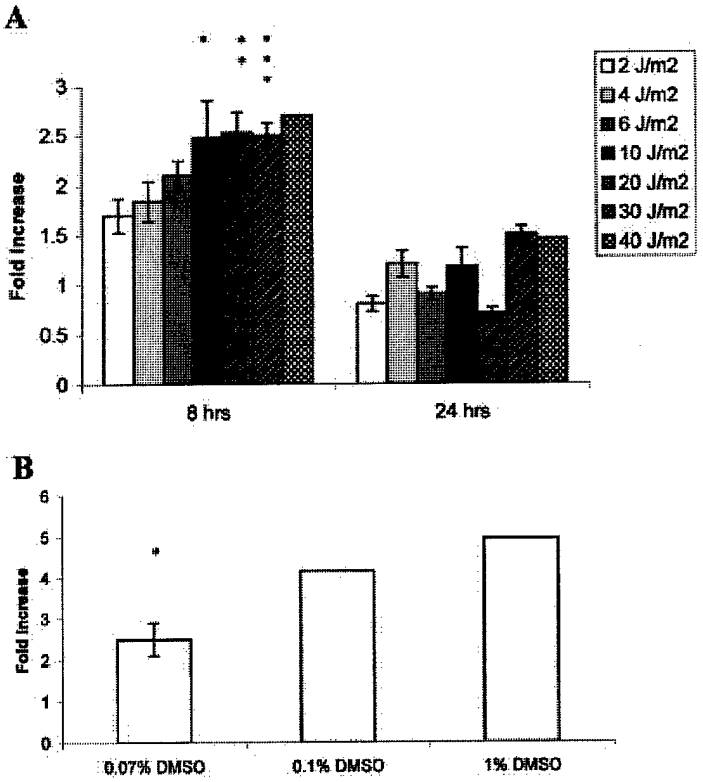

FIG. 1. UV- and DMSO-induced telomerase activity in the SW626 cell line. By using absorbance values of the control samples as the denominator, fold increase of the treated samples was determined. (A) UV-induced telomerase activity was measured at 0, 8, and 24 h, following 2, 4, 6, 10, 20, 30, and 40 J/m^2 of UV in triplicate samples of SW626 cells by PCR–ELISA, and the results are expressed as fold increase ± SE. Treatment with 40 J/m^2 was performed only in duplicate. $^*P \leq 0.04$, $^{**}P \leq 0.03$, $^{***}P \leq 0.03$ B. DMSO-induced telomerase activity in SW626 cells measured 8 h following 0.07, 0.1, and 1.0% DMSO. Triplicate samples were assayed for telomerase activity and the results expressed as fold increase ± SE, $^*P \leq 0.005$.

RESULTS

UV and DMSO elevate basal telomerase activity and induce de novo telomerase activity. For each cell line employed, sublethal doses of UV were determined using a cell proliferation assay following various doses of UV (data not shown). With the control absorbance value set as 1 (not shown), a peak 2.75-fold elevation in endogenous telomerase activity was observed 8 h following treatment of SW626 cancer cells with 2–40 J/m^2 (Fig. 1A). We found that telomerase induction was related to dose, with telomerase activity increasing linearly between 2 and 10 J/m^2, then plateauing between 10 and 40 J/m^2, with almost a 3-fold increase following treatment with 40 J/m^2 (Fig. 1A). Like UV treatment, DMSO-induced telomerase activity peaked at 8 h following treatment (Fig. 1B). While DMSO treatment also appeared to be dose-related, the increases in telomerase activity ranged from 2.5-fold

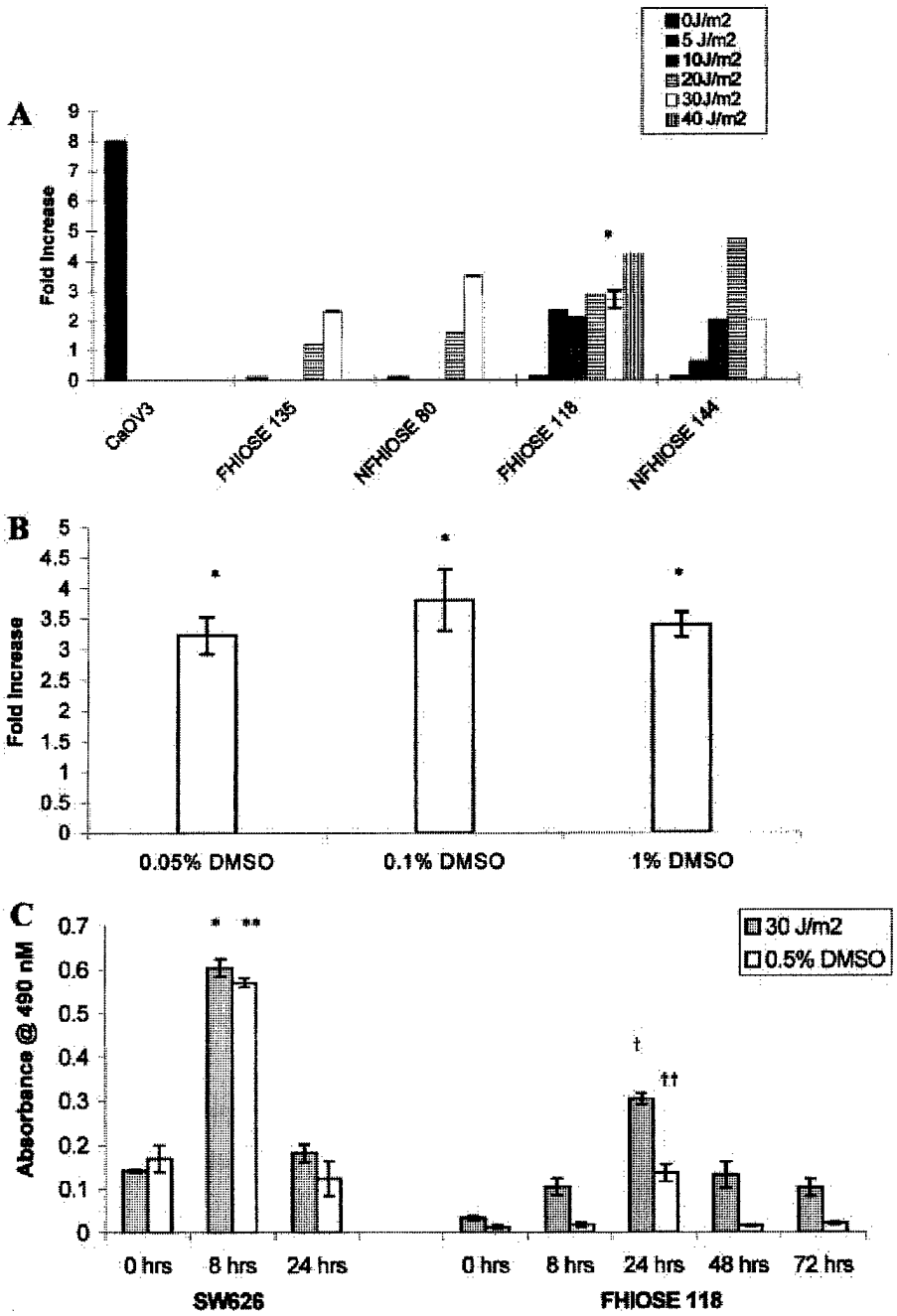

FIG. 2. UV- and DMSO-induced telomerase activity in the IOSE cell lines. Values were expressed as a fold increase above control levels, with the control value used as the denominator for the determination of fold increase of the treated samples. (A) UV-induced telomerase activity in IOSE cells was measured at 24 h following 0, 5, 10, 20, 30, and 40 J/m^2 UV from duplicate samples of IOSE cell lines, and the results are expressed as fold increase. SE was only determined in one instance. *$P \leq 0.02$. (B) DMSO-induced telomerase activity in FHIOSE 118 cells following 0.05, 0.1, and 1.0% DMSO. Triplicate samples were assayed for telomerase activity, and the results are expressed as fold increase ± SE. *$P \leq 0.005$. (C) Comparison of UV- and DMSO-induced telomerase activity in SW626 cells at 0, 8, and 24 h and in FHIOSE 118 cells at 0, 8, 24, 48, and 72 h following treatment with 30 J/m^2 UV or 0.5% DMSO. *$P \leq 0.009$, **$P \leq 0.001$, †$P \leq 0.02$, ‡$P \leq 0.003$.

with 0.07% DMSO to 5-fold with 1% DMSO (Fig. 1B). Further, both UV- and DMSO-induced telomerase activity was transient in SW626 cells, since the elevated levels of telomerase activity returned to near basal levels within 24 h of UV or DMSO treatment (Figs. 1A and 2C).

We expanded our studies to determine whether UV or DMSO could activate telomerase activity in telomerase-negative, nontumorigenic ovarian epithelial cell lines. In contrast to the highly telomerase-positive CaOV3 ovarian cancer cells, which served as our internal control, the levels of telomerase detected for all four

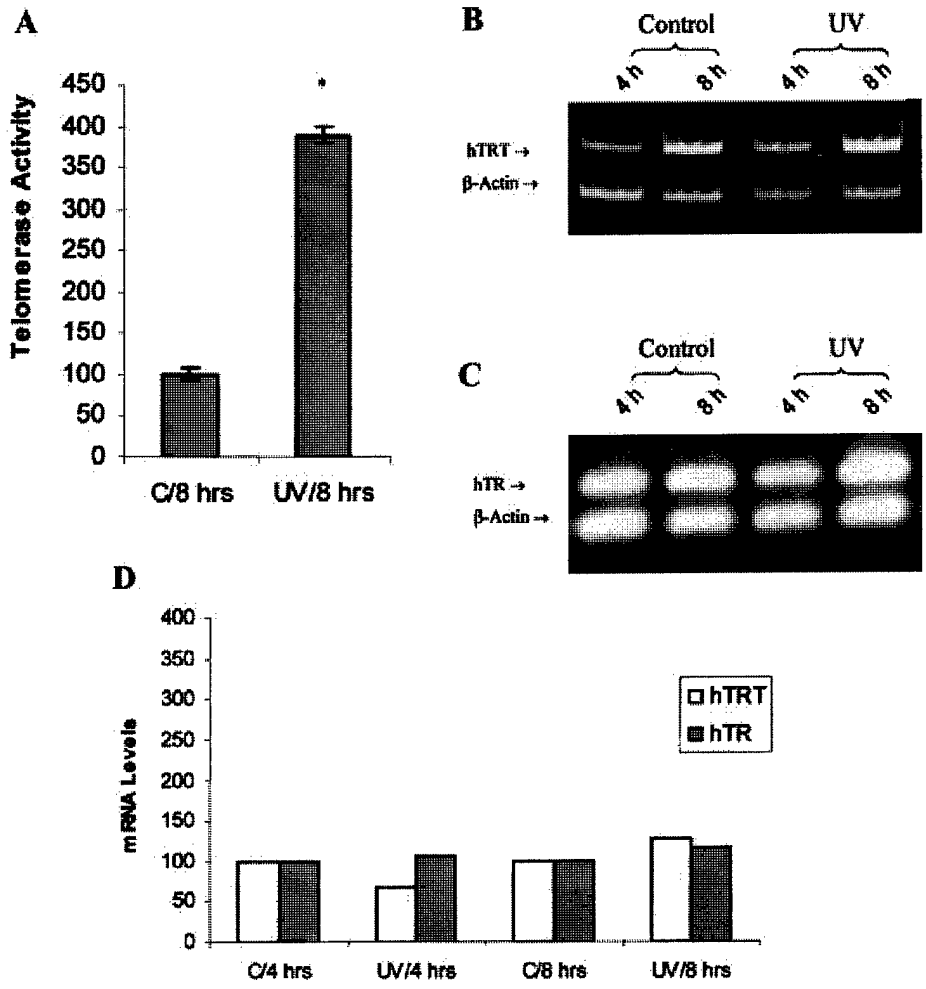

FIG. 3. hTRT and hTR mRNA levels following UV-induced telomerase activity in SW626 cells. (A) SW626 cells were treated with 30 J/m^2 UV and assayed for telomerase activity by PCR–ELISA 8 h following treatment. Samples were assayed in triplicate and expressed as mean percentage of control ± SE (C, control). *$P \leq 0.005$. (B) PAGE following RT–PCR revealed a 145-bp hTRT product and a 98-bp β-actin product. (C) PAGE following RT–PCR revealed a 103-bp hTR product and a 98-bp β-actin product. (D) Graphical representation of hTRT and hTR mRNA net intensities in treated and untreated samples relative to β-actin mRNAs.

untreated IOSE cell lines examined (FHIOSE 135, FHIOSE 118, NFHIOSE 80, and NFHIOSE 144) were similar to the assay background levels, defined by the assay as "telomerase-negative." Following UV or DMSO treatment, all IOSE cell lines exhibited detectable levels of telomerase activity (Figs. 2A and 2B). Although a dose-dependent relationship was not observed in IOSE cells, treatment with 5–40 J/m^2 UV induced a 1.5- to 5.5-fold increase in telomerase activity (Fig. 2A). Treatment with varying doses of DMSO resulted in a 2.7- to 2.9-fold induction of telomerase activity in a dose-independent manner (Fig. 2B).

While UV or DMSO induced telomerase activity in both SW626 and FHIOSE118 cell lines, the absolute amount of telomerase activity induced was dramatically different between the two cell lines. In order to demonstrate this difference in telomerase levels, SW626 and FHIOSE 118 cell lines were treated with UV or DMSO and absorbance values of the assay were compared directly (Fig. 2C). Treatment with 0.5% DMSO or 30 J/m^2 UV resulted in a 3- to 4-fold increase over endogenous telomerase activity in SW626 cells by 8 h (Fig. 2C), returning to control levels by 24 h. In FHIOSE 118 cells, a 9- to 10-fold increase in telomerase activity was observed at 24 h following UV or 0.5% DMSO treatment (Fig. 2C). In contrast to SW626 cells, FHIOSE 118 cells demonstrated maximal induction of telomerase activity at 24 h following UV or DMSO treatment (Fig. 2C). Telomerase activity was lost in FHIOSE 118 cells within 48 h following treatment with DMSO. Forty-eight hours following UV treatment, the FHIOSE 118 cells continued to exhibit progressively decreasing telomerase levels. These data suggest that UV- and DMSO-induced telomerase activity may be a transient event in both cell lines.

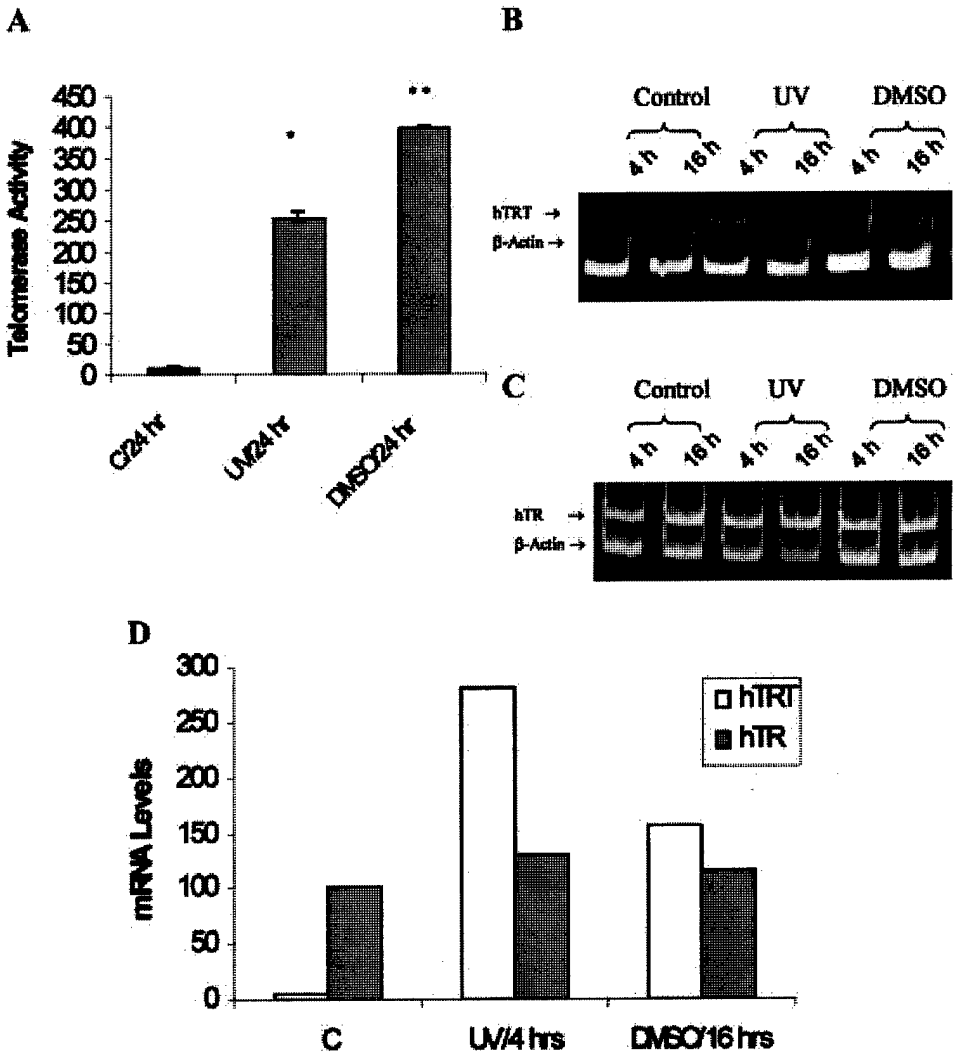

FIG. 4. hTRT and hTR mRNA levels following UV- and DMSO-induced telomerase activity in FHIOSE 118 cells. (A) FHIOSE 118 cells were treated with 30 J/m^2 UV or 0.5% DMSO and assayed for telomerase activity by PCR–ELISA 24 h following treatment. Telomerase activity was expressed as a fold increase above control (C) levels, with the control value used as the denominator for the determination of fold increase of the treated samples. Samples were assayed in triplicate and expressed as mean percentage of control ± SE. $^*P \leq 0.05$, $^{**}P \leq 0.005$ (B) PAGE following RT–PCR revealed a 145-bp hTRT product and a 98-bp β-actin product in UV and DMSO-treated samples at 4 and 16 h, respectively, but only the 98-bp β-actin product was detected in untreated FHIOSE 118 cells. (C) PAGE following RT–PCR revealed a 103-bp hTR product and a 98-bp β-actin product in all samples. (D) Graphical representation of hTRT and hTR net intensities in treated and untreated samples relative to β-actin mRNAs.

UV and DMSO induction of telomerase activity is transcription-dependent in FHIOSE 118 cells but not in SW626 cells. To examine the role of transcriptional control in UV- and DMSO-induced telomerase activity, RT–PCR was employed to determine the relative levels of the telomerase catalytic component (hTRT) and the RNA component (hTR) mRNA in SW626 and FHIOSE 118 cells (Figs. 3 and 4). SW626 cells were irradiated with 30 J/m^2 and assayed for telomerase activity at 8 h following treatment (Fig 3A). A 3.75-fold increase in telomerase activity was detected between the control sample and the UV-irradiated sample. With telomerase levels peaking at 8 h in the SW626 cells, it was necessary to begin collection points prior to those times in order to detect a change in mRNA levels. RT–PCR and PAGE analyses of parallel cultures revealed a 145- and a 98-bp band, representing the hTRT and β-actin products, respectively, at 4 and 8 h (Figs. 3B and 3C). However, despite the 3.75-fold increase in telomerase activity, no significant differences in the relative levels of hTRT transcript were observed between the control and UV-irradiated samples (Figs. 3B and 3D). RT–PCR and PAGE analyses also showed that there appeared to be no difference in the level of hTR transcript between the control and UV-irradiated SW626 samples (hTR transcript levels never exceeding 10% that of the controls) (Figs. 3C and 3D).

RT–PCR was similarly utilized to determine the rel-

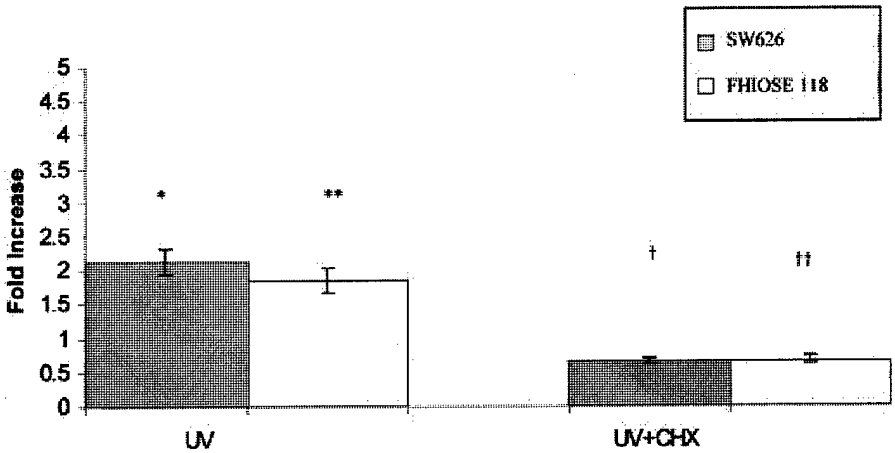

FIG. 5. Effect of cycloheximide on UV-induced telomerase activity. SW626 and FHIOSE 118 cells were treated with 40 μM CHX, as described under Methods and Materials, treated with 30 J/m^2 UV, and subsequently assayed for telomerase activity. Absorbance values of the control samples were used as the denominator for the determination of fold increase of the treated samples. Results are triplicate samples expressed as fold increase ± SE. $^*P \leq 0.05$, $^{**}P \leq 0.02$, $\dagger P \leq 0.03$, $\ddagger P \leq 0.008$.

ative levels of hTRT and hTR mRNAs in FHIOSE 118 cells following UV- and DMSO-induced *de novo* telomerase activity (Fig. 4). FHIOSE 118 cells were assayed for telomerase activity 24 h following treatment with either 30 J/m^2 UV or 0.5% DMSO. Both UV and DMSO treatment resulted in a significant induction of telomerase activity (Fig. 4A). UV caused a 2.5-fold induction of telomerase activity and treatment with DMSO resulted in a 4-fold induction compared to control levels. With telomerase levels requiring 24 h for maximal activity in the FHIOSE 118 cells, 4- and 16-h time points were selected in order to detect a change in mRNA levels. While the 98-bp band representing β-actin mRNA product was seen in all lanes, RT–PCR and PAGE analysis did not detect hTRT transcripts in untreated FHIOSE 118 cells (Figs. 4B and 4D). However, the 145-bp hTRT mRNA band was clearly visible in UV- and DMSO-treated samples collected at 4 and 16 h following treatment (Figs. 4B and 4D). Maximal hTRT mRNA levels were noted at 4 h following UV, having increased 280% above the control levels. hTRT mRNA levels steadily declined by 16 h following UV, decreasing to 156% (Fig. 4B) above the control levels. For DMSO-treated samples, peak hTRT transcript levels were observed at 16 h following treatment, having increased 300% above control levels. RT–PCR and PAGE analyses also revealed the constitutive expression of the 103- and 98-bp bands representing hTR and β-actin mRNA products, respectively, in treated and untreated FHIOSE 118 cells (Fig. 4C). Unlike hTRT mRNA levels, there was no apparent change in the levels of hTR transcripts between the control and UV-irradiated or DMSO-treated samples (Figs. 4C and 4D). Therefore, it appears that induced telomerase activity was preceded by an increase in hTRT transcript levels in FHIOSE 118 cells.

Induction of telomerase activity is partly dependent upon protein synthesis in SW626 and FHIOSE 118 cells. To determine whether protein synthesis was required for telomerase induction, the protein synthesis inhibitor CHX was used to inhibit *de novo* protein synthesis in SW626 and FHIOSE 118 cells following treatment with 30 J/m^2 UV (Fig. 5). In this set of experiments, UV-treated SW626 and FHIOSE 118 cells demonstrated an approximate twofold elevation in telomerase activity following treatment with 30 J/m^2 (Fig. 5). CHX inhibited UV-induced telomerase activity, decreasing SW626 telomerase levels to 50% of the control level by 8 h. However, CHX did not suppress basal telomerase activity in SW626 cells (data not shown). Treatment with CHX partially inhibited UV-induced telomerase activity in FHIOSE 118 cells but did not completely abolish UV-induced telomerase activity. Specifically, CHX suppressed UV-induced telomerase activity in FHIOSE 118 cells by 60%. For fold increase calculations, absorbance values of the control samples were used as the denominator for the determination of fold increase of the treated samples.

PI3K activity is increased following UV and DMSO treatment. Although UV is known to activate PI3K [27–33], it was necessary to determine whether DMSO was capable of activating PI3K. To compare the levels of PI3K activation between UV and DMSO, PI 3-kinase assays were performed following treatment of SW626 and FHIOSE 118 cells with UV or DMSO, respectively (Fig. 6). Since kinases are rapidly activated, samples were collected 30 min following treatment, when the kinases were maximally activated. Here, SW626 cells were treated with 30 J/m^2 UV and collected at 30 min following treatment. Similarly, FHIOSE 118 cells were treated with DMSO and collected 30 min following

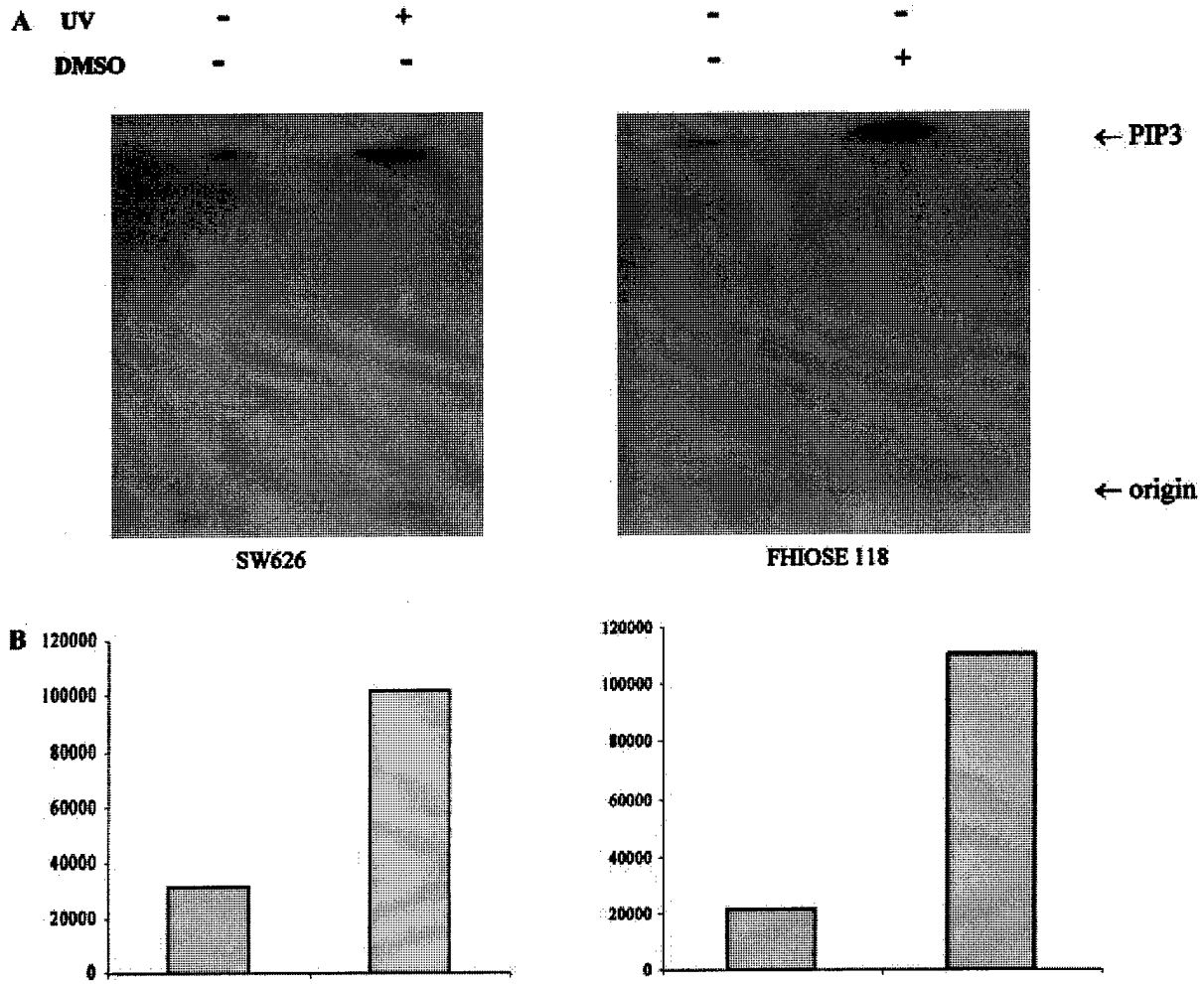

FIG. 6. PI 3-kinase activation precedes UV- and DMSO-induced telomerase activity. (A) SW626 cells were treated with 30 J/m^2 UV and FHIOSE 118 cells were treated with 0.05% DMSO. Cells were collected and assayed for PI3K activity as described under Methods and Materials. The conversion of PI(4,5)P2 to PI 3-phosphate was determined by autoradiography. (B) ImageQuant software was used to quantify the PI 3-phosphate product. Graphical representation depicts band intensities as measured by the imaging software.

treatment. Lysates were then assayed for PI3K activity as previously described. In SW626 cells, PI3K activity increased 3-fold following UV treatment. Following treatment with DMSO, FHIOSE 118 cells demonstrated a 5-fold increased in PI3K activity, demonstrating an increase in PI3K activity above that seen with 30 J/m^2 UV. To the best of our knowledge, this is the first demonstration of DMSO-induced PI3K activity.

Inhibition of PI3K suppresses hTRT transcription in FHIOSE 118 cells but not in SW626 cells. In order to confirm the involvement of the PI3K signaling pathway for telomerase regulation in our model system, the PI3K inhibitors WM and LY were used. Using absorbance values of the control samples as the denominator for the determination of fold increase of the treated samples, a 2-fold elevation in telomerase activity was observed following treatment with 30 J/m^2 UV or 0.5% DMSO in SW626 cells (Fig. 7A). However, LY and WM suppressed UV- and DMSO-induced telomerase activity to below basal levels. The SW626 cells treated only with PI3K inhibitors demonstrated a 40% reduction in the basal telomerase activity. FHIOSE 118 cells treated with 30 J/m^2 UV or 0.5% DMSO demonstrated 3.3- and a 2-fold inductions, respectively, in telomerase activity above background (Fig. 7B). As with SW626 cells, LY blocked UV- and DMSO-induced telomerase activity in FHIOSE 118 cells by 250 ($P \leq 0.01$) and 175% ($P \leq 0.05$), respectively. It should be noted that treatment with either WM or LY did not result in increased cell death, as determined by trypan blue exclusion (data not shown).

To determine if PI3K inhibitors abrogated telomerase induction by affecting the transcription of telomerase components, RT–PCR for hTRT and hTR mRNAs was employed following treatment with LY in SW626 (Fig. 8) and FHIOSE 118 (Fig. 9) cells. Treatment with 10 μM LY suppressed 60% of the basal telomerase activity in SW626 cells (Fig. 8A). However, LY-medi-

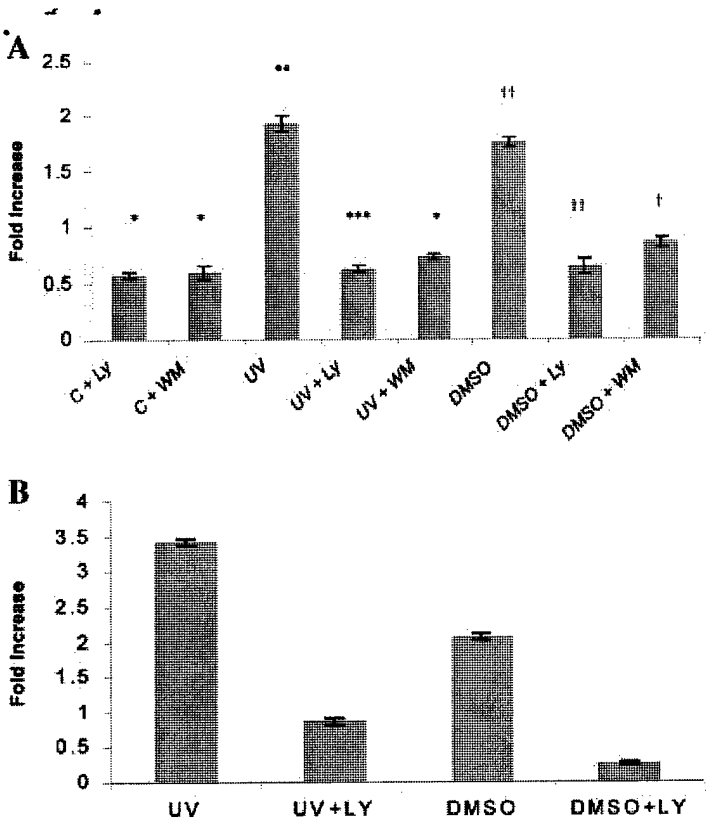

FIG. 7. Inhibition of PI3K inhibits UV or DMSO induction of telomerase activity. Telomerase activity is expressed as fold increase using the absorbance valued of the control samples as the denominator for the determination of fold increase of the treated samples. (A) SW626 cells were treated with either 10 μM LY or 100 nM WM, as described under Methods and Materials, exposed to either 30 J/m^2 UV or 0.5% DMSO, and subsequently assayed for telomerase activity. Results are triplicate samples expressed as fold increase ± SE. $^*P \leq 0.02$, $^{**}P \leq 0.002$, $^{***}P \leq 0.004$, $\dagger P \leq 0.001$, $\dagger\dagger P \leq 0.003$ (B) FHIOSE 118 cells were treated with 10 μM LY, treated with either 30 J/m^2 UV irradiation or 0.5% DMSO, and subsequently assayed for telomerase activity. $P \leq 0.05$ for UV + Ly and DMSO + Ly compared to their respective controls. Results are triplicate samples expressed as fold increase ± SE.

ated suppression of telomerase activity in SW626 cells was not associated with any notable change in either hTRT or hTR mRNA levels, as determined by RT–PCR and PAGE analyses (Figs. 8B–8D).

Similarly, FHIOSE 118 cells were treated with UV in order to induce telomerase activity. UV treatment resulted in a 3.4-fold induction of telomerase ($P \leq 0.02$). Parallel cultures were treated with 10 μM LY in addition to UV. Ten micromolar LY suppressed UV-induced telomerase activity approximately 250% in FHIOSE 118 cells ($P \leq 0.01$) (Fig. 9A), and this inhibition of telomerase induction was associated with decreased levels of hTRT mRNA (Figs. 9B–9D). This confirms a role for PI3K in telomerase regulation.

Chemotherapeutic agents do not induce telomerase activity. To determine if DNA damage could result in the induction of telomerase activity, SW626 cells were treated with sublethal doses of a variety of chemotherapeutic agents and then assayed, in duplicate, for telomerase activity (Fig. 10). Sublethal doses of all chemotherapeutic agents were determined by performing a cell proliferation assay for each agent at the various concentrations used (data not shown). Changes in telomerase activity observed following treatment with 25 or 100 μm CP, 10, 50, or 100 μm ET, and 1, 10, or 25 μm CB (Fig. 10) were not statistically significant ($P > 0.05$). Thus, assays were not performed in triplicate, and the standard errors are not shown.

DISCUSSION

Not only is telomerase expressed in over 90% of ovarian cancers, telomerase activity has also been shown to correlate well with ovarian tumor aggressiveness or stage [4, 25, 37–40]. Although information about telomerase regulation is incomplete, various studies propose a complex model of regulation [1, 10, 12, 13]. Given the important clinical connection between telomerase and ovarian cancer, it is imperative to devise a model system in which telomerase activity can be manipulated and studied. UV and DMSO were deemed to be potential modulators of telomerase activity in ovarian epithelial cell lines based upon our current understanding of telomerase regulation and ovarian neoplastic progression. Specifically, since Ueda *et al.* reported increased telomerase activity following UV exposure of normal skin [53] and Hande *et al.* reported a dose-dependent increase in telomerase activity following UV treatment in CHO cells [54], we sought to determine whether UV could induce telomerase activity in ovarian epithelial cell lines. Further, in contrast to carcinomas of most other tissues that de-differentiate with neoplastic progression, ovarian cancer progression is associated with the acquisition of morphological features characteristic of the endocervix, endometrium, and oviduct owing to their common embryonic origin [55–57]. For example, features associated with a more differentiated epithelial phenotype in ovarian neoplasia include expression of E-cadherin, activin receptors, $\alpha_v\beta_3$-integrin, vitronectin, and CA125, products normally associated with the oviduct, endometrium, and endocervix [56–60]. Bearing this in mind, we also attempted to determine whether DMSO, a commonly employed differentiation-inducing agent, could induce telomerase activity by mimicking the neoplastic progression of ovarian surface epithelial cells. While we examined our cells for markers of differentiation such as E-cadherin expression, no morphological changes were detected (data not shown). However, DMSO was capable of inducing PI 3-kinase activity as well as telomerase activity. In contrast to our findings, others have reported telomerase suppression associated with DMSO-induced cellular differentiation

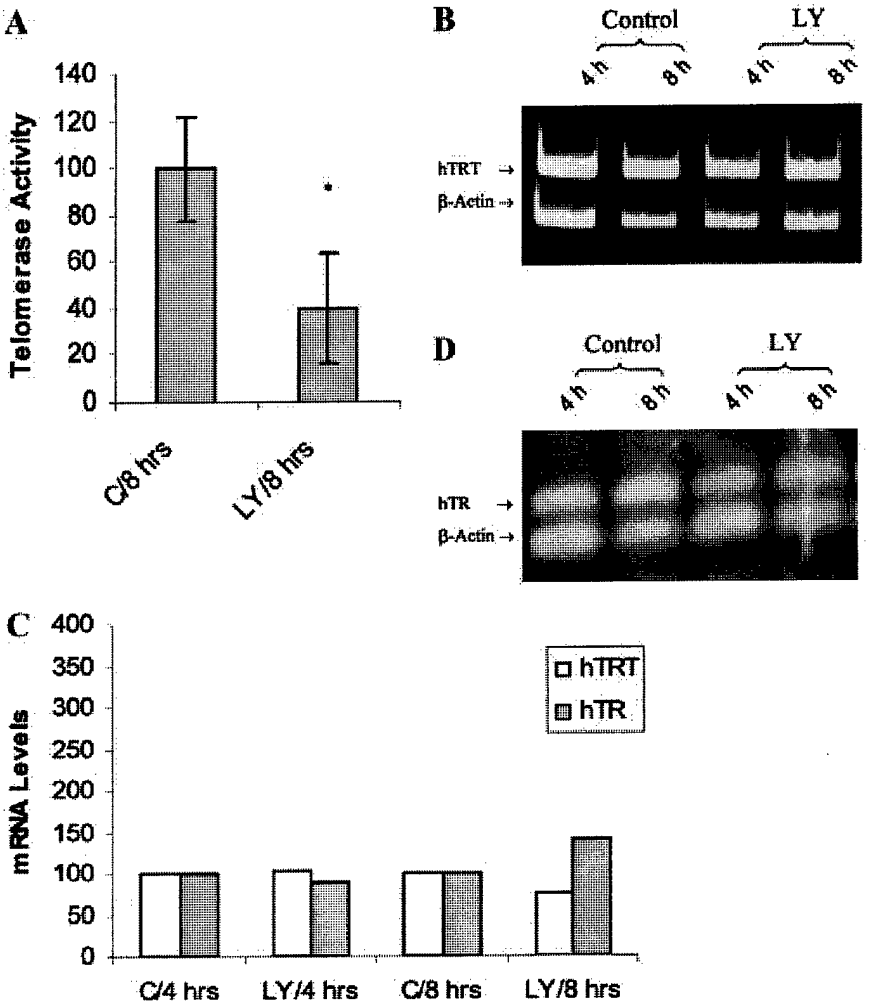

FIG. 8. hTRT and hTR mRNA levels in SW626 cells following LY-mediated suppression of telomerase activity. (A) SW626 cells were treated with 10 μM LY and assayed for telomerase activity by PCR–ELISA at 8 h. Telomerase activity is expressed as fold increase using the absorbance values of the control (C) samples as the denominator for the determination of fold increase of the treated samples. Samples were assayed in triplicate and expressed as mean percentage of control ± SE. *$P \leq 0.005$. (B) PAGE following RT–PCR revealed a 145-bp hTRT product and a 98-bp β-actin product in all samples. β-Actin was used as an internal control. (C) PAGE following RT–PCR revealed a 103-bp hTR product and a 98-bp β-actin product in all samples. β-Actin was used as an internal control. (D) Graphical representation of hTRT or hTR net intensity relative to β-actin mRNAs.

and/or G_0/G_1 cell cycle arrest in a variety of leukemia, colon carcinoma, teratocarcinoma, and lymphoma cells [15, 16]. Discrepancies in the effects of DMSO on telomerase activity observed by these authors and those in the present study may be due to variation in treatment methodologies. While other studies employ high-dose DMSO treatment for periods of days, our treatment involved much lower doses for a 2-h treatment period.

We have developed a culture model system in which we have shown, for the first time, that UV or DMSO can transiently induce *de novo* telomerase activity in previously telomerase-negative human ovarian cell lines as well as elevate endogenous telomerase activity in ovarian cancer cells. Using the very sensitive telomerase ELISA assay, changes in telomerase activity were represented graphically as fold increase, where the absorbance values of the control samples were used as the denominator for the determination of fold increase of the treated samples. We found a dose-related elevation or induction of telomerase activity, following UV or DMSO treatment, in telomerase-positive SW626 and telomerase-negative FHIOSE 118 ovarian cell lines, respectively. UV-induced telomerase activity appeared to be greater in magnitude in FHIOSE 118 cells than in the SW626 cell line and may reflect the fact that FHIOSE cells are normally telomerase negative. In addition, we also found differences in the kinetics of UV- or DMSO-induced telomerase activity between telomerase-positive and -negative cell lines. SW626 cancer cells exhibited maximally elevated telomerase levels at 8 h following UV or DMSO, while maximal

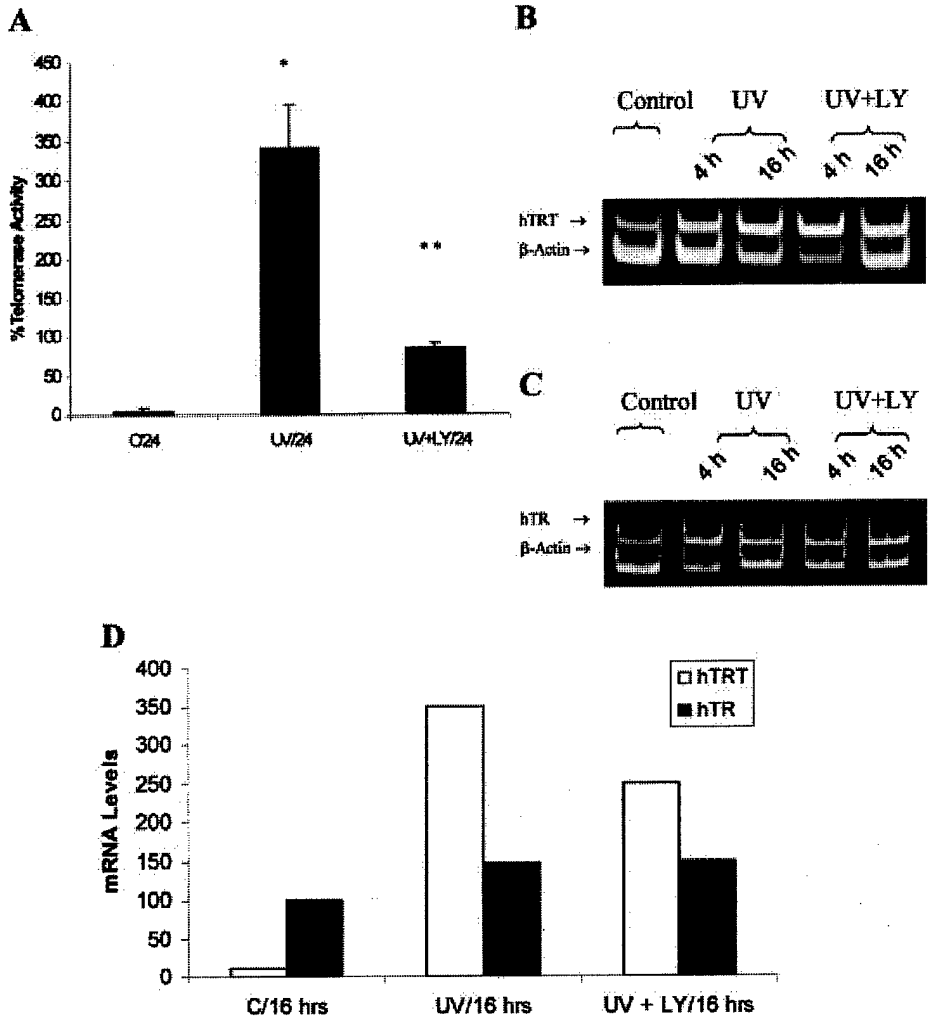

FIG. 9. hTRT and hTR mRNA levels in FHIOSE 118 cells following LY-mediated suppression of UV-induced telomerase activity. (A) FHIOSE 118 cells were treated with 30 J/m^2 UV. Telomerase activity is expressed as fold increase using the absorbance values of the control (C) samples as the denominator for the determination of fold increase of the treated samples. Triplicate samples were assayed for telomerase activity by PCR–ELISA and expressed as mean percent of control ± SE. *$P \leq 0.02$, **$P \leq 0.01$ (B) PAGE following RT–PCR revealed a 145-bp hTRT product in the treated samples. RT–PCR also revealed a 98-bp β-actin product in all samples. β-Actin was used as an internal control. (C) PAGE following RT–PCR revealed a 103-bp hTR product and a 98-bp β-actin product in all samples. β-Actin was used as an internal control. (D) Graphical representation of hTRT or hTR net intensity relative to β-actin mRNAs.

telomerase induction in FHIOSE 118 cells occurred only at 24 h following treatment. Furthermore, we found that UV- or DMSO-induced telomerase activity was transient in all cell lines examined. Induced telomerase activity progressively decreased in FHIOSE cells by 48 h after UV or DMSO treatment, while induced telomerase activity in SW626 cells returned to controls levels by 24 h after treatment. These results are in agreement with previous observations reporting a strong association between telomerase activity with sun exposure [53], a UV dose-dependent induction of telomerase activity in CHO cells [54], and restoration of basal telomerase levels within 24 h following withdrawal of DMSO [61].

We used our model system to identify some of the components that may regulate telomerase activity. We found that SW626 cells constitutively expressed both hTR and hTRT mRNA. DMSO- or UV-induced elevation of telomerase was not associated with an increase in either hTRT or hTR transcription, suggesting that SW626 may constitutively express maximal levels of hTRT and hTR mRNAs. In contrast, UV- or DMSO-induced *de novo* telomerase activity in FHIOSE 118 cells required transcription of hTRT, but not hTR, mRNA. This finding may reflect the lag time for telomerase induction in these cells. Again, our results are in agreement with those of others, which suggest that while cells often constitutively express the hTR component of telomerase, hTRT expression is the rate-limiting factor in telomerase activity [1, 3–5, 51, 62].

Furthermore, we also showed that protein synthesis was required for *de novo* telomerase activity in

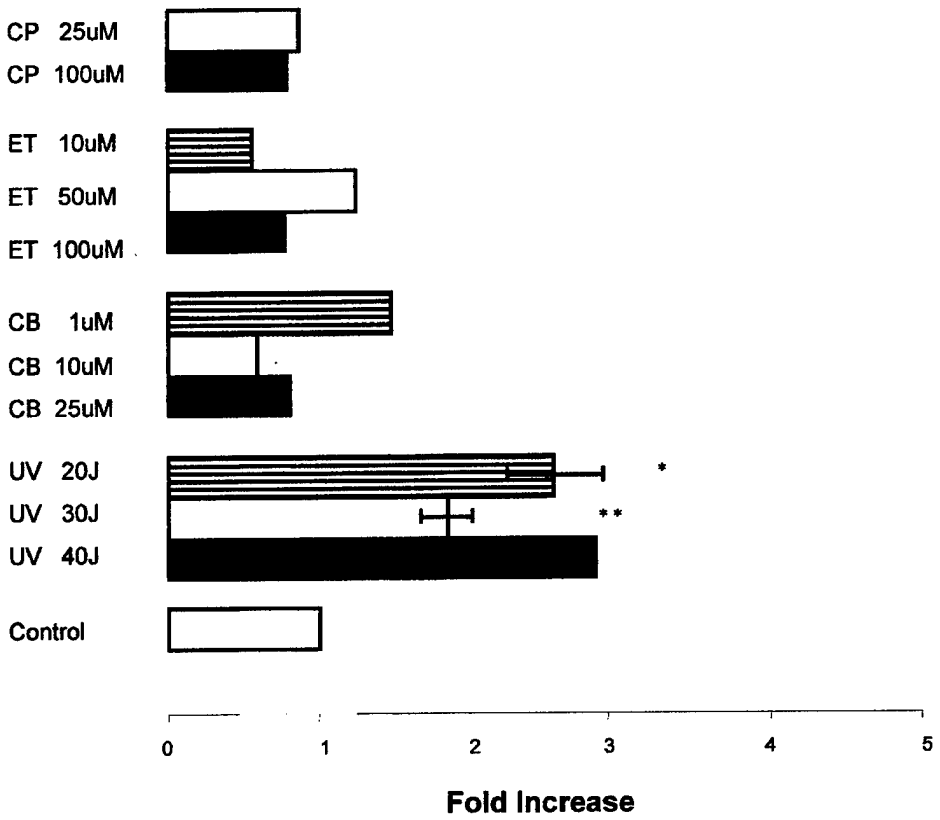

FIG. 10. Effect of chemotherapeutic agents on telomerase activity in SW626 cells. Cells were treated as described under Methods and Materials. Telomerase activity is expressed as fold increase using the absorbance values of the control samples as the denominator for the determination of fold increase of the treated samples. Only 20- and 30-J/m^2 samples were assayed in triplicate, the remainder were in duplicate. Fold increases in telomerase activity are shown ± SE. $^*P \leq 0.04$, $^{**}P \leq 0.03$.

FHIOSE 118 cells as well as for elevating endogenous telomerase activity in SW626 cells. CHX inhibited UV-induced telomerase activity in FHIOSE 118 cells by 60% and diminished telomerase activity in SW626 cells to 50% of control levels. The inability of CHX to completely abolish telomerase activity may be due to the 24-h half-life of telomerase [63]. While the exact role of protein synthesis in telomerase regulation remains unclear, CHX is a general protein synthesis inhibitor and its inhibitory action may prevent synthesis of the telomerase catalytic component, hTRT, and/or associated proteins such as tankyrase, TLP1, or as of yet unidentified components of the telomerase complex. Therefore, cells without prior detectable telomerase activity, such as FHIOSE 118 cells, would be required to assemble telomerase from *de novo* transcription, translation, and assembly of its component parts.

PI3K can be activated by exogenously applied cellular stress [64] and it has been implicated in telomerase regulation [20]. Akt, a down-stream target of PI3K, has also been implicated in the activation of telomerase by direct phosphorylation of the hTRT catalytic component [24]. We also examined the relative contribution of PI3K to telomerase regulation in our model system. PI 3-kinase assays revealed that PI3K levels were increased following UV exposure, as previously reported [27–33]. In addition, we report here, for the first time, that DMSO can increase PI3K activity levels. Using WM or LY, both specific inhibitors of PI3K, we were able to demonstrate inhibition of UV- or DMSO-induced telomerase activity in both SW626 and FHIOSE 118 cells. This inhibition of telomerase activity through inhibition of PI3K occurred independent of changes in hTRT and hTR transcription levels. These results suggest that, perhaps, at least initiation of telomerase activity in FHIOSE 118 cells may involve the same signal transduction pathway that regulates cancer cells. These findings also suggest that there may be an additional component in telomerase regulation, such as posttranslational modification by phosphorylation of one or more of the telomerase components.

While the exact mechanism(s) of telomerase activation is not completely understood, the TTAGGG telomeric repeat sequence has been shown to be a hot spot for chromosomal breakage [65, 66], a target for UV-induced pyrimidine dimers, as well as a target for CP- or CB-induced intrastrand d(GpG) and d(ApG) cross-links [67, 68]. Consequently, commonly used chemotherapeutic agents might increase telomerase activity and potentially undermine chemotherapeutic efficacy.

Therefore, we also sought to determine whether chemotherapeutic agents would induce telomerase activity in our model system. We treated SW626 cells for a period of time previously found to be sufficient to induce intrastrand cross-links without causing significant cellular lethality [68]. We were unable to suppress endogenous telomerase activity in SW626 cancer cells with comparable doses of chemotherapeutic agents, as has been reported elsewhere [69]. This may be attributed to inherent differences in the cell types used as well as the length of exposure to the drug employed. Taken together, our results suggest that enhanced telomerase activity following cellular perturbation is specific to certain exogenous applied agents. Further, our results support current therapeutic modalities for ovarian cancer, since use of these chemotherapeutic agents does not appear to promote telomerase activity, decreasing the probability of telomerase-promoted tumor cell survival.

In summary, we have developed a novel and useful culture model system, for elevating telomerase activity in a telomerase-positive cell line, and, most importantly, for demonstrating *de novo* telomerase activity in a telomerase-negative cell line using UV or DMSO. The exact mechanism of telomerase regulation in our model system is not completely known, and clearly further studies are warranted. It appears that UV and DMSO induction of telomerase activity begins a signaling cascade, originating at the cellular membrane, via membrane perturbation caused by UV or DMSO [70–74]. This, in turn, may activate telomerase activity through PI3K, possibly by phosphorylation of the hTRT component. While maintenance of telomerase levels may require only protein synthesis and post-translational modifications such as phosphorylation, initiation of *de novo* telomerase activity requires hTRT transcription and protein synthesis. Lastly, the lack of telomerase induction by chemotherapeutic agents in our model system will allow for additional studies aimed at determining clinically feasible mechanisms to suppress telomerase in cancer cells without reducing the efficacy of conventional chemotherapy.

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Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer

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We previously demonstrated that AKT2, a member of protein kinase B family, is activated by a number of growth factors via Ras and PI 3-kinase signaling pathways. Here, we report the frequent activation of AKT2 in human primary ovarian cancer and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase (PI 3-kinase)/Akt pathway. *In vitro* AKT2 kinase assay analyses in 91 ovarian cancer specimens revealed elevated levels of AKT2 activity (>3-fold) in 33 cases (36.3%). The majority of tumors displaying activated AKT2 were high grade and stages III and IV. Immunostaining and Western blot analyses using a phospho-ser-473 Akt antibody that detects the activated form of AKT2 (AKT2 phosphorylated at serine-474) confirmed the frequent activation of AKT2 in ovarian cancer specimens. Phosphorylated AKT2 in tumor specimens localized to the cell membrane and cytoplasm but not the nucleus. To address the mechanism of AKT2 activation, we measured *in vitro* PI 3-kinase activity in 43 ovarian cancer specimens, including the 33 cases displaying elevated AKT2 activation. High levels of PI 3-kinase activity were observed in 20 cases, 15 of which also exhibited AKT2 activation. The remaining five cases displayed elevated AKT1 activation. Among the cases with elevated AKT2, but not PI 3-kinase activity (18 cases), three showed down-regulation of PTEN protein expression. Inhibition of PI 3-kinase/AKT2 by wortmannin or LY294002 induces apoptosis in ovarian cancer cells exhibiting activation of the PI 3-kinase/AKT2 pathway. These findings demonstrate for the first time that activation of AKT2 is a common occurrence in human ovarian cancer and that PI 3-kinase/Akt pathway may be an important target for ovarian cancer intervention. *Oncogene* (2000) **19,** 2324–2330.

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Akt/PKB represents a subfamily of the serine/threonine protein kinases (Bellacosa *et al.*, 1991; Jones *et al.*, 1991a, b; Cheng *et al.*, 1992; Konishi *et al.*, 1995; Nakatani *et al.*, 1999). Three members of this family, AKT1/PKBα, AKT2/PKBβ and AKT3/PKBγ have been identified. Akt is activated by a variety of stimuli, including growth factors, protein phosphatase inhibitors, and stress (Franke *et al.*, 1995; Burgering *et al.*, 1995; Shaw *et al.*, 1998; Liu *et al.*, 1998). Activation of Akt and AKT2 by growth factor is mediated by PI 3-kinase (Franke *et al.*, 1995; Meier *et al.*, 1997; Liu *et al.*, 1998). Active Ras and Src have also shown to activate Akt and AKT2 and this activation is blocked by wortmannin, a PI 3-kinase inhibitor, indicating that Ras and Src also mediate the activation of Akt and AKT2 and are located upstream of PI 3-kinase (Datta *et al.*, 1996; Liu *et al.*, 1998). Several lines of evidence suggest that PI 3-kinase regulates Akt activation through the following mechanism: the product of PI 3-kinase, phosphatidylinositol-3,4,5-trisphosphate, binds to the pleckstrin homology (PH) domain of Akt after growth factor stimulation, resulting in recruitment of Akt to the cell membrane. A conformational change of Akt follows, which allows residues Thr-308 and Ser-473 to be phosphorylated by upstream kinases, PDK-1 and PDK2 or ILK, respectively (Alessi and Cohen, 1998; Delcommenne *et al.*, 1998). Several downstream targets of Akt, each of which contains the Akt phosphorylation consensus sequence R-X-R-X-X-S/T-F/L, have been identified (Alessi and Cohen, 1998), pointing to the possible mechanisms by which Akt promotes cell survival and blocks apoptosis. One such target is GSK3. Akt phosphorylates GSK3 and leads to inactivation of GSK3, accumulation of β-catenin, and activation of *Myc* transcription. There is also evidences that Akt phosphorylates the proapoptotic proteins BAD, caspase-9 and transcription factor FKHRL1, resulting in reduced binding of BAD to Bcl-X_L and inhibition of caspase-9 protease activity and Fas ligand transcription (Datta *et al.*, 1997; del Peso *et al.*, 1997; Cardone *et al.*, 1998; Brunet *et al.*, 1999). Recent studies demonstrated that PTEN/MMAC1 tumor suppressor and SHIP, tyrosine and inositol phosphatases, dephosphorylate phosphatidylinositol-3,4,5-triphos-phate, thus, inhibiting the PI 3-kinase/Akt signaling pathway (Stambolic *et al.*, 1998; Aman *et al.*, 1998).

Several members within the PI 3-kinase/Akt pathway, including PI 3-kinase, PTEN, AKT2, and β-catenin, have been implicated in human neoplasms (Alessi and Cohen, 1998). Overexpression of p85, the regulatory subunit, or p110, the catalytic subunit, of PI 3-kinase is able to transform cells (Chang *et al.*, 1997; Jimenez *et al.*, 1998). Alterations of PI 3-kinase have also been detected in a number of human malignancies (Shayesteh *et al.*, 1999; Phillips *et al.*, 1998). Mutation and/or down regulation of the *Pten* are frequently observed in endometrial carcinoma, glioblastoma, breast cancer and prostate carcinoma (Stambolic *et*

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al, 1998). We and others have previously demonstrated alterations of *AKT2* at DNA, mRNA and/or protein level in several types of human malignancy (Cheng *et al*., 1992, 1996; Bellacosa *et al*., 1995; Ruggeri *et al*., 1998). In particular, amplification/overexpression of *AKT2* has been detected in 10–20% of ovarian carcinomas and pancreatic cancers. However, increased activation of AKT2 in human tumors has not been demonstrated previously.

In this report, we show that kinase activity of AKT2 is frequently elevated in human primary ovarian tumor. *In vitro* AKT2 kinase assays were performed in 91 primary human ovarian tumors, including 34 serous cystadenocarcinomas, four mucinous cystadenocarcinomas, 25 papillary serous adenocarcinomas, 10 endometrioid adenocarcinomas, two borderline tumors, five granulosa cell tumors and 11 other rare types of tumors including adenosarcoma, thecoma, fibroma, and mesodermal mixed tumor. Lysates from tumor specimens containing equivalent amounts of protein were precleared and incubated with anti-AKT2 antibody, which specifically reacts with AKT2, in the presence of protein-A : protein-G beads. The immunoprecipitates were subjected to *in vitro* kinase assay using Histone H2B as the substrate. *In vitro* kinase assays were carried out three times for each specimen. Average reading of the kinase activity threefold higher than that in normal ovarian tissue was considered elevated AKT2 activity. The results revealed an elevated level of AKT2 kinase in 33 specimens (36.3%), including 15 serous cystadenocarcinomas (44%), 14 papillary serous adenocarcinomas (56%) and four other types of tumor including one adenosarcoma, two malignant clear cell tumors, and one mixed mullerian tumor. Examples of AKT2 kinase activity in ovarian cancer are shown in Figure 1. Interestingly, the majority of cases exhibiting activation of AKT2 were serous adenocarcinomas (29/33). None of endometrioid, borderline, mucinous, and granulosa cell tumors exhibited elevated AKT2 kinase activity.

To examine whether variations in AKT2 protein expression level contributes to the level of AKT2 activity observed in ovarian tumor specimens, we analysed its expression by Western blot analyses using a specific anti-AKT2 antibody. A high level of AKT2 protein was detected in 42 of 91 cases (46%). The 33 cases that exhibited elevated AKT2 activity displayed variable levels of AKT2 protein, 25 of which expressed AKT2 at high or moderate levels (Figure 1 and Table 1). These results were also confirmed by immunohistochemical staining of the tumor tissue section (data not shown).

Figure 1 Elevated level of AKT2 kinase activity in ovarian cancer. Top panel: *In vitro* kinase assays of AKT2 immunoprecipitated from 15 frozen representative tumor specimens. Frozen tissues from ovarian carcinoma was mechanically smashed in liquid nitrogen and lysed by a Tissue Tearor in a lysis buffer (Liu *et al*., 1998). Lysates were incubated with anti-AKT2 antibody in the presence of protein A-protein G (2 : 1) agarose beads for 2 h at 4°C. Following extensive wash, immunoprecipitates were subjected to *in vitro* kinase assay (Liu *et al*., 1998). Histone H_2B was used as the exogenous substrate. Each experiment was repeated three times. The relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a Phosphorimager. AKT2 is activated in cases T2, T5, T7, T10, T13, and T15. Middle panel: Western blot analysis of AKT2 expression in ovarian carcinoma. The blot was reprobed with anti-β-actin antibody (Bottom panel)

Previous studies demonstrated that phosphorylation of Thr-308 and Ser-473 of Akt1 is required for its full activation. Upon stimulation of PI 3-kinase by growth factors, Akt1 becomes phosphorylated at these two residues. It has been shown that AKT2 is activated by phosphorylation of the equivalent residues (Thr-309 and Ser-474) (Alessi and Cohen, 1998). The homology of Akt1 to AKT2 and AKT3 is 90.4% and 87.8% at the amino acid level, respectively (Cheng *et al*., 1992; Nakatani *et al*., 1999), and the sequence of the serine-473 phosphorylation site of Akt1 is highly conserved in AKT2 and AKT3 (Figure 2a).

Anti-phospho-Ser473 Akt antibody has widely been used to identify Akt1 activation. To determine whether such an antibody can be used to detect AKT2 activation in ovarian cancer specimens by Western blotting and immunostaining, we tested whether a phospho-Ser473 Akt antibody reacted with phosphorylated AKT2. HA-AKT2 expression construct was transfected to COS-7 cells. After serum-starvation overnight and IGF-1 stimulation for 10 min, HA-AKT2 was immunoprecipitated with anti-HA monoclonal antibody. The immunoprecipitates were separated by SDS–PAGE and probed with phospho-Ser473 Akt antibody. The results showed that the antibody against phospho-Ser473 Akt strongly reacted with phospho-Ser 474 of AKT2 (Figure 2b).

We next examined AKT2 phosphorylation by Western blot analyses of AKT2 immunoprecipitates with phospho-Ser473 Akt antibody in 43 ovarian tumor specimens including the 33 cases with and 10 cases without elevated AKT2 kinase activity. A sheep anti-AKT2 antibody was used to incubate with equal amount of protein from ovarian tumor lysates in the presence of protein-A and protein-G agarose. After extensive wash with the lysis buffer, the AKT2 immunoprecipitates were separated by SDS–PAGE and the blots were probed with the rabbit polyclonal phospho-Ser473 Akt antibody (New England Biolab). Phosphorylated AKT2 was detected only in ovarian tumors with elevated AKT2 kinase activity; for example, see data from tumors T2, 5, 7, 10 and 13, which exhibit high AKT2 kinase activity, shown in Figure 2c. These data confirm the results obtained from *in vitro* AKT2 kinase assay and indicate that AKT2 activity is regulated by phosphorylation at Ser474 in human primary tumors.

Table 1 AKT2 activation and clinical stage

		AKT2 kinase activity		*AKT2 protein level*	
Stage	n	*Normal*	*High*	*Low*	*High/moderate*
I	7	7	0	5	2
II	11	11	0	6	5
IIIA	5	5	0	1	4
IIIC	53	27	26	31	22
IV	15	8	7	6	9

Due to the fact that stromal tissues approximately account for 20~30% of the tumor specimens used in this study, we examined whether activation of AKT2 is derived from tumor cells or stromal tissues by immunostaining the tumor paraffin sections with phospho-Ser473 Akt antibody. We first tested if the phospho-Ser473 Akt antibody is capable of recognizing phosphorylated AKT2 by immunohistochemistry. Cell paraffin blocks were prepared from serum-starved and serum-starved/EGF-stimulated OVCAR-3 cells that overexpress AKT2 (Cheng *et al.*, 1992). Phospho-Ser473 antibody was used to detect phosphorylated AKT2 in the section from these blocks by immunohistochemical means. Strong positive staining was observed in EGF-stimulated but not serum-starved OVCAR-3 cells (panel 1~2 in Figure 3a). Phosphorylation status of AKT2 in these cells was confirmed by Western blot analysis with phospho-S473 Akt antibody (lanes 1 and 2 in Figure 3b).

Immunostaining of the tumor paraffin sections with phospho-Ser473 antibody was performed in 43 ovarian cancer specimens including 33 cases exhibiting elevated AKT2 kinase activity and 10 cases without AKT2 activation. Positive staining of tumor cells was observed in 38 cases including 33 with and five without AKT2 activation detected by *in vitro* kinase assay and Western blot analyses. The fact that phospho-Ser473 Akt antibody recognizes Akt1, AKT2 and possible AKT3 suggests that activation of AKT1 or AKT3 present in these five cases, which may be due to different expression levels of three isoforms of Akt. Immunohistochemical staining of paraffin tissue sections with anti-AKT1 and -AKT2 antibodies revealed that AKT1 but not AKT2 is highly expressed in these five specimens (Figure 3c). Moreover, *in vitro* kinase assay showed that AKT1 kinase activity is elevated in these specimens (Figure 3d, and also see case 8 in Figures 1 and 4). Unfortunately, no good AKT3 antibody is commercially available at present time, therefore, we could not determine if AKT3 protein is altered in these specimens. Strikingly, phosphorylated Akt localizes to cytoplasm and cell membrane. Even in EGF-stimulated OVCAR-3 cells, activated Akt stays in cytoplasm and cell membrane. No nuclear staining was observed (panels 2 and 4 of Figure 3a).

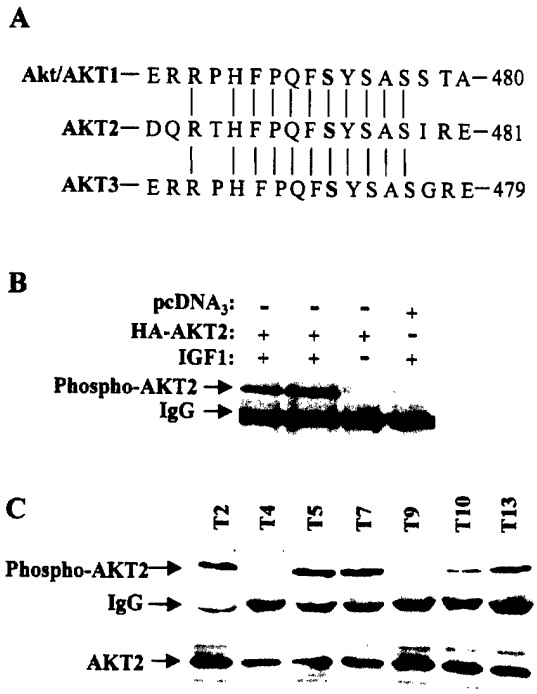

Figure 2 Phospho-Akt-Ser473 antibody recognizes phosphorylation form of AKT2 that is detected in ovarian cancer. (**a**) Comparison of C-terminal amino acid sequence of AKT1 and AKT2 and AKT3. Phosphorylation of serine-473 (AKT1) is well conserved in AKT2 (Ser-474) and AKT3 (Ser-472). (**b**) Western blot analysis of HA-AKT2 immunoprecipitates from COS-7 cells transfected with pcDNA$_3$-HA-AKT2 or pcDNA$_3$ vector alone. After transfection, the cells were serum-starved overnight and stimulated with IGF-1 for 10 min (lanes 1 and 2 from left) prior to harvesting cells. Immunoprecipitation was carried out with anti-HA monoclonal antibody and separated by SDS–PAGE. The blot was detected with Phospho-Akt-Ser473 antibody. (**c**) Western blot analysis of phosphorylation of AKT2 in ovarian cancer specimens. The tumor lysates were incubated with anti-AKT2 antibody. The resulting immunoprecipitates were separated by SDS–PAGE and detected with polyclonal phospho-Ser473 Akt antibody (upper panel) or polyclonal anti-AKT2 antibody (lower panel)

One mechanism that could result in an increased activation of AKT2 is an up-regulation of PI 3-kinase. PI 3-kinase is a heterodimer composed of a p85-regulatory and a p110-catalytic subunit. Three isoforms of p85 and p110 have been cloned, namely p85α, p85β, p85γ, p110α, p110β and p100γ. Recent studies demonstrated transforming activity of p110 and p85 and frequent activation PI 3-kinase in colon and ovarian carcinoma cell lines (Chang *et al.*, 1997; Jimenez *et al.*, 1998; Shayesteh *et al.*, 1999; Phillips *et al.*, 1998). To examine PI 3-kinase activity in tumor tissues, immunoprecipitation with a pan-p85 specific antibody was performed in 43 ovarian cancer cases, including 33 with and 10 without AKT2 activation (Klippel *et al.*, 1993). Following extensive wash, the immunoprecipitates were subjected to *in vitro* PI 3-kinase assay using L-α-phosphatidylinositol-4,5-bis phosphate (PI-4,5-P$_2$) or L-α-phosphatidylinositol-4-phosphate (PI-4-P$_1$) as substrates. The conversion of PI-4,5-P$_2$ to PI-3,4,5-P$_3$ or PI-4-P$_1$ to PI-3,4-P$_2$ was determined by autoradiography and quantitated by Phosphorimager. Elevated PI 3-kinase activity was detected in 20 ovarian cancer specimens, 15 of which exhibited AKT2 activation and the other five displayed AKT1 activation (Figures 3 and 4). Since p110α has been shown to be over-expressed and activated in ovarian cancer cell lines (Shayesteh *et al.*, 1999), we examined whether increased levels of activity observed in our primary ovarian tumor specimens resulted from increased levels of p110 expression. PI 3-kinase was immunoprecipitated with an anti-p85 antibody from equal amounts of tumor extract protein. The immunoprecipitates were then separated on a SDS–PAGE, transferred to a membrane and probed with a specific anti-p110α antibody. Figure 4 shows a correlation between p110α expression levels and PI 3-kinase activity in tumor samples. Quantitative analysis by a BioImager (Genomic Solutions Inc.) revealed that the levels of p110α expression in the tumors with PI 3-kinase activation are 2~4-folds higher than that in the specimens showing no elevated PI 3-kinase, except T12 that exhibited high level of p110α protein but no significant PI 3-kinase activation. These data indicate that increases of PI 3-kinase activity contribute to AKT activation in human primary ovarian carcinoma.

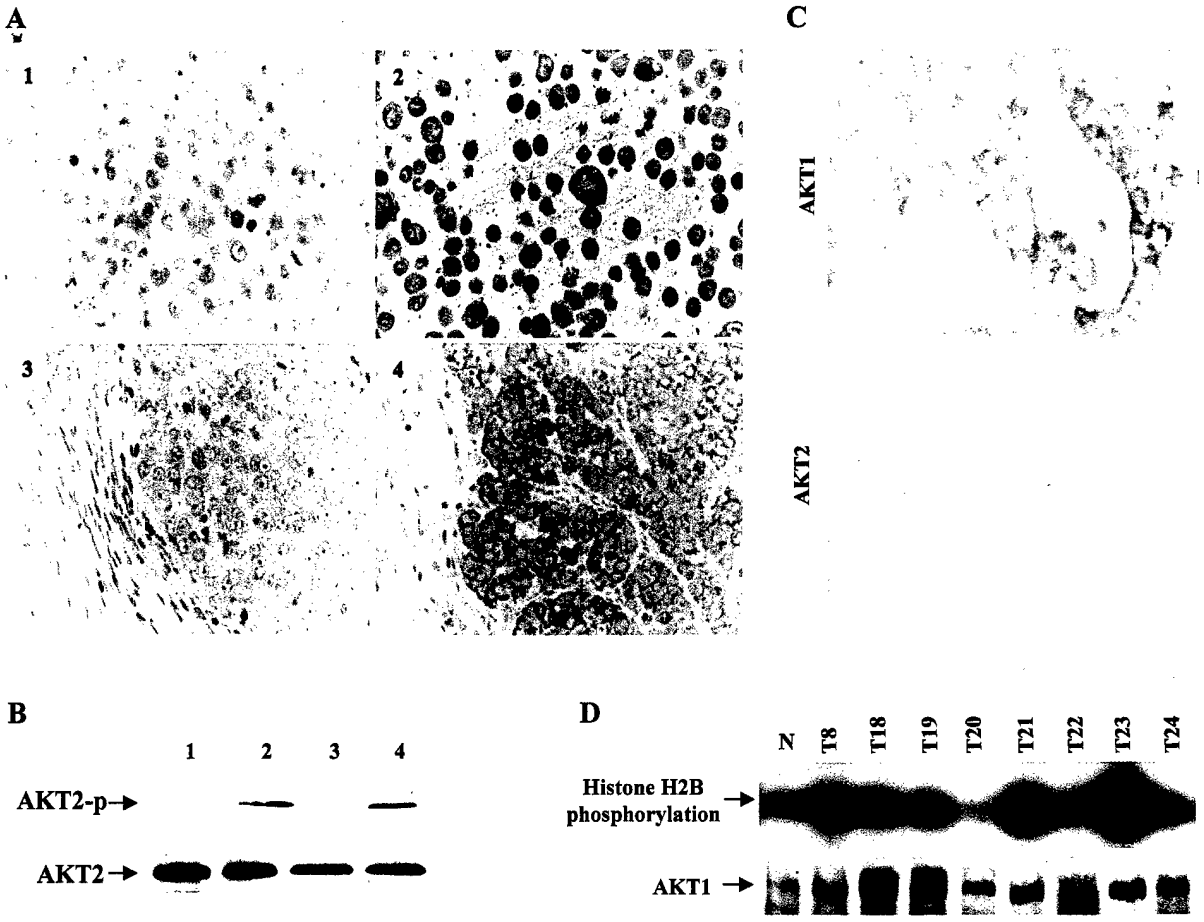

Figure 3 Phosphorylated Akt detected by immunostaining localizes to cell membrane and cytoplasm of ovarian tumor cells. The paraffin sections were subjected to antigen retrieval by boiling in a microwave and then incubated in a blocking solution and an avidin/biotin blocking kit (Vector). The primary antibody to phospho-S473 Akt (Upstate Biotechnology) was applied at a dilution of 1:200. After incubation, the slides were treated with biotinylated rabbit anti-goat immunoglobulin and streptavidin and biotinylated alkaline phosphatase. (**a**) Sections of paraffin blocks prepared from serum-starved (1), EGF-stimulated OVCAR3 cells (2) and 2 primary ovarian carcinoma specimens (3~4) were stained with phospho-Ser473 antibody. No staining was observed in serum starved OVCAR3 cells and a specimen without elevated AKT2 kinase activity (1 and 3), whereas strong reaction with phospho-Ser473 antibody is seen in EGF-stimulated OVCAR3 cells and a tumor specimen exhibiting AKT2 activation. (**b**) Western blot analysis of the AKT2 immunoprecipitates from the cells and the tissues that are used for immunostaining in Figure 3a. The filter was detected with phospho-Ser473 antibody (upper panel) and anti-AKT2 antibody (lower panel). (**c**) Immunohistochemical staining of an ovarian cancer specimen with anti-AKT1, and -AKT2 antibodies. (**d**) *In vitro* kinase assay (upper panel) and Western blot (bottom panel) of AKT1 immunoprecipitates from frozen tumor specimens. Results represent three independent experiments. AKT1 is activated in cases T8, T18, T19, T21, and T23

Another mechanism shown to result in activation of Akt is inactivation or loss of expression of PTEN. Inactivating mutations of the *Pten* tumor suppressor gene, on chromosome 10q23, have been described in prostate, endometrial and ovarian endometrioid carcinomas (Obata *et al*., 1998). Previous studies have also observed down-regulation of PTEN protein in prostate cancer (Wu *et al*., 1998; McMenamin *et al*., 1999). Therefore, we examined whether down-regulation of PTEN is associated with AKT2 activation in ovarian cancer. We analysed PTEN expression in 18 ovarian cancer specimens displaying elevated AKT2 activity by Western blot. A large reduction in PTEN protein expression was observed in three cases, two of which were serous cystadenocarcinomas and one was a papillary serous adenocarcinoma (Figure 5). In these cases, we did not detect either mutations or low mRNA level of the *Pten* gene (data not shown). Interestingly, we failed to observe elevated AKT2 kinase activity in 10 endometrioid adenocarcinomas examined, even though PTEN mutations have most frequently been detected in this type of ovarian tumor. These results suggest that down-regulation of PTEN protein expression, either by translational or post-translational changes, may be a factor in elevating AKT2 activation in non-endometrioid ovarian cancer.

We next examined the relationships between activation of AKT2 and tumor stage and grade. The results are presented in Tables 1 and 2. High levels of AKT2 activity were seen in stages III and IV (33/68, 48.5%) but not stages I and II. The incidence of AKT2 activation increased with increasing grade. High levels of AKT2 kinase activity were detected in 52.9% of grades III and IV tumors, whereas activation of AKT2 was observed in only 15% of grades I and II tumors. These data indicate that activation of AKT2 in ovarian carcinoma is associated with late stage and high-grade tumors, and suggest an increase in the activation of AKT2 kinase as ovarian cancer progresses to a more aggressive phenotype.

It has been shown that Akt induces cell survival and suppresses the apoptotic death of a number of cell types induced by a variety of stimuli, including growth factor

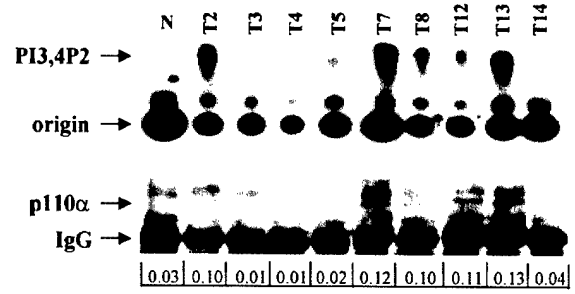

Figure 4 Activation of PI 3-kinase in human ovarian cancer specimens. *In vitro* PI 3-kinase assay of the anti-p85 immunoprecipitates from 12 frozen ovarian cancer specimens. The tumor tissue lysates were immunoprecipitated with pan-p85 antibody (Santa Cruz). Following wash, the presence of PI 3-kinase activity in immunoprecipitates was determined by incubating the beads with reaction buffer. Phospholipids were extracted and separated by thin-layer chromatography as previously described (Jiang *et al*., 2000). The conversion of PI-4,5-P_2 to PI 3-phosphate and PI-4-PI to PI-3,4-P_2 was determined by autoradiography and quantitated by using a Phosphorimager. Elevated levels of PI 3-kinase activity (top panel) and p110α protein (middle panel) are detected in cases 2, 7, 8, 12, and 13. The expression levels of p110α are quantified by a BioImager. The density of each band is indicated at the bottom

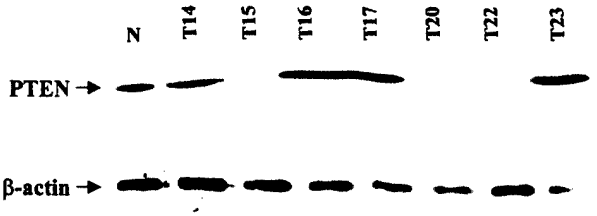

Figure 5 Down regulation of PTEN in human ovarian carcinoma. Western blot analyses of the tumor tissue lysates with anti PTEN (upper) and β-actin (bottom) antibody. No detectable level of PTEN was observed in three cases

Table 2 AKT2 activation and grade

		AKT2 kinase activity	
Grade	n	*Normal*	*High*
1	21	17	4
2	19	17	2
3	49	24	25
4	2	0	2

withdrawal, cell-cycle discordance and loss of cell adhesion. To assess the influence of PI 3-kinase/AKT activation on the cell growth of human ovarian cancer cells, we have performed *in vitro* kinase assay and observed activation of AKT2 and PI 3-kinase in 2 of 5 ovarian cancer cell lines (Figure 6a). If PI 3-kinase/Akt pathway is crucial for survival in such ovarian tumor cells, blocking the activity of PI 3-kinase/Akt is expected to inhibit the cell growth and/or induce apoptosis. To test this hypothesis, three ovarian cancer cell lines with or without PI 3-kinase/AKT2 activation were treated with PI 3-kinase inhibitors, wortmannin (200 nM) or LY294002 (40 μM), or vehicle (DMSO), for 12 h in a medium containing 1% fetal calf serum. Those cell lines exhibiting elevated levels of PI 3-kinase and AKT2 activity underwent apoptosis after treatment with wortmannin or LY294002, whereas no apoptosis was detected in the cell line (e.g., A2780) without PI 3-kinase/AKT2 activation (Figure 6).

In this study, we have demonstrated frequent activation of AKT2 kinase in human primary ovarian cancer by *in vitro* kinase assay, Western blot and immunohistochemical staining. Phosphorylated AKT2 has a cell membrane and cytoplasmic but not nuclear localization. Increased PI 3-kinase activation is observed in the majority of cases displaying AKT2 activation. High levels of AKT2 protein expression are also frequently detected in ovarian cancer specimens. Down regulation of PTEN was observed in non-endometrioid ovarian tumors, which may contribute to AKT2 activation. Moreover, activation of AKT2 is associated with high grade and late stage ovarian cancer and direct inhibition of PI 3-kinase/Akt pathway induces apoptosis in ovarian cancer cell lines exhibiting activation of PI 3-kinase and AKT2.

Previous studies showed that alteration of oncogene could occur at DNA, mRNA, protein, or enzymatic level. An extensive review of the topic indicates that the frequency of oncogene amplification in primary tumors is about 5–38%, depending on tumor type, stage, and grade as well as the individual gene (Brison, 1993). The incidence of overexpression of some oncogenes in certain tumors is even higher due to the mechanism of enhanced transcription or translation, and mRNA or protein stabilization (Brison, 1993; Devilee and Cornelisse, 1994; Berns *et al*., 1995). We and others have previously shown alterations of *AKT2* at DNA and mRNA levels in ~12% and ~25% of ovarian carcinoma, respectively (Cheng *et al*., 1992; Bellacosa *et al*., 1995). In this study, we demonstrate activation of AKT2 kinase in 36.3% of primary ovarian tumors with highest frequency (49%) in serous adenocarcinomas. Moreover, we have also observed overexpression of AKT2 protein in 46% of ovarian cancer specimens examined. These data, as compared to *AKT2* alteration at DNA and RNA levels, indicate that activation and overexpression of AKT2 protein by aberrant translational regulation and posttranslational modification of AKT2 is a more common occurrence in human ovarian cancer. The fact that overexpression of wild type AKT2 in NIH3T3 cells results in malignant transformation (Cheng *et al*., 1997) suggests that activation and overexpression of AKT2 in human ovarian carcinoma could play an important role in the development of this malignancy.

Several studies demonstrated that ectopically expressed Akt1 and AKT2, following growth factor stimulation, initially locates to cell membrane and then translocates to the nucleus (Andjelkovic *et al*., 1997; Meier *et al*., 1997). This is thought to allow Akt mediated phosphorylation of nuclear transcription factors, such as Forkhead proteins and CREB (Brunet *et al*., 1999; Kops *et al*., 1999; Du and Montminy, 1998). In contrast, in primary ovarian tumor specimens examined, we found that phosphorylated AKT2 localizes to cell membrane and cytoplasm but not the nucleus. Moreover, activated endogenous AKT2 in AKT2-overexpressing OVCAR cells was also localized to cell membrane and cytoplasm only. These conflicting observations may be due to the fact that ectopic overexpression of Akt affects its subcellular localization.

We and others have previously documented that AKT2 is a downstream target of PI 3-kinase and is activated by a number of growth factors (Meier *et al*.,

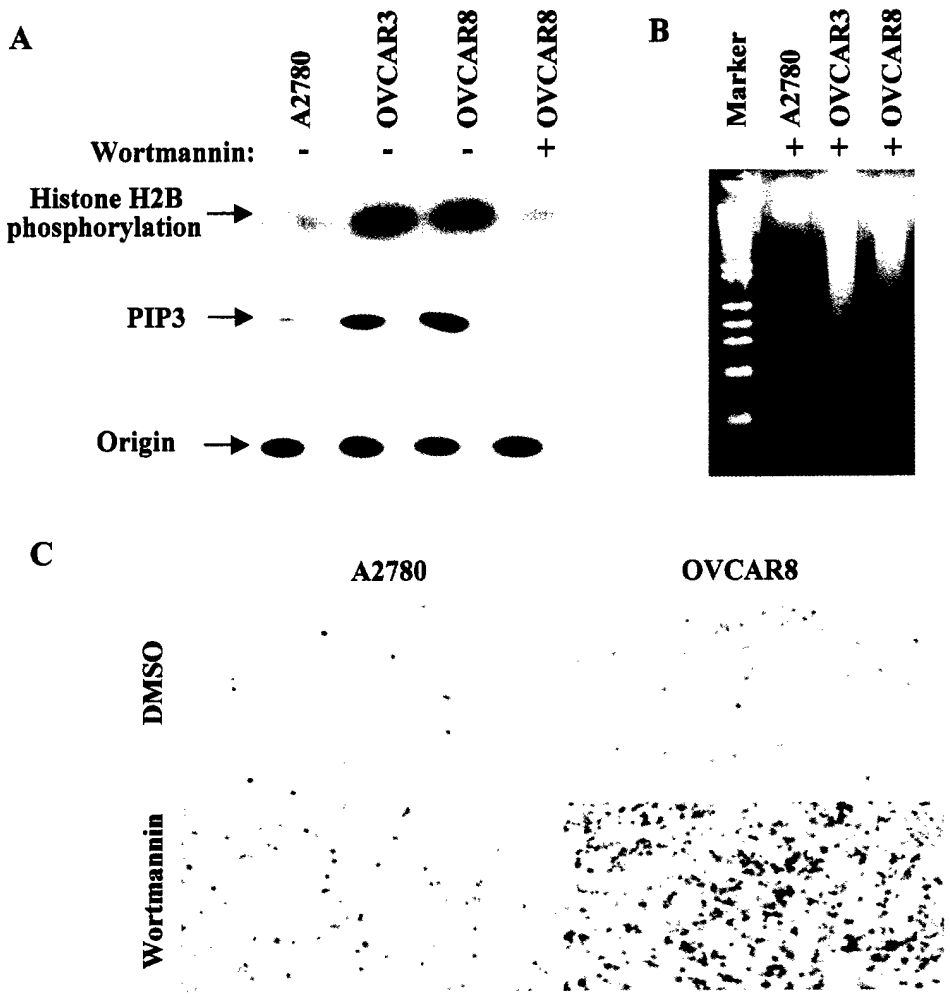

Figure 6 Inhibition of PI 3-kinase/AKT2 activity induces apoptosis in PI 3-kinase/AKT2-activating ovarian cancer cell lines. (**a**) *In vitro* Akt and PI 3-kinase assay of the immunoprecipitates from A2780, OVCAR3 and OVCAR8 cells. AKT2 (upper panel) and PI 3-kinase (bottom panel) are activated in OVCAR3 and OVCAR8 cells. (**b**) and (**c**) DNA fragmentation and Tunel assay showing that Wortmannin blocks activity of PI 3-kinase and AKT2, and induces apoptosis in OVCAR3 and OVCAR8 cells. The cells were seeded into 60 mm dishes and grown for 24 h and then treated with wortmannin (200 nM) or LY294002 (40 μM) for 12 h. Apoptosis was determined by TdT-mediated dUTP nick end labeling (Tunel) using an *in situ* cell death detection kit (Boehringer). To detect DNA fragmentation, cellular DNA was prepared using the DNA kit (Qiagen). The DNA was analysed on 1.5% agarose gel and visualized by ethidium bromide staining

1997; Liu *et al*., 1998). Active Ras and Src significantly induce AKT2 activation (Liu *et al*., 1998). Recent studies have shown that Akt activity is regulated by *PTEN*, which reduces intracellular levels of PI-3,4,5,-P_3 in cells by converting PI-3,4,5-P_3 to PI-4,5-P_2 and, thus, inhibits PI 3-kinase/Akt signaling pathway (Stambolic *et al*, 1998; Aman *et al*., 1998). Therefore, AKT2 activation in human ovarian cancer may result from; (a) PI 3-kinase activation; (b) *Pten* mutation; (c) overexpression of AKT2; (d) alterations of growth factor receptor, such as overexpression or mutation of EGFR; (e) *ras* mutation; or (f) active mutation of the *AKT2* gene. In this report, we document that nearly half of cases with AKT2 activation (15/33) display PI 3-kinase activation, which supports a recent observation of PI 3-kinase activation in ovarian cancer cell lines (Shayesteh *et al*., 1999). Down regulation of PTEN was detected in three non-endometrioid cases of ovarian tumors. Interestingly, we did not observe either lack of PTEN expression or AKT2 activation in all 10 endometrioid adenocarcinomas examined, implying that the *Pten* may not be mutated in these specimens. We have also performed single strain conformation polymorphism analyses of the tumors carrying activated AKT2. No *AKT2* mutation was observed. The cases exhibiting AKT2 activation express variable levels of AKT2 protein, suggesting that expression of AKT2 protein is required for activation of AKT2 in ovarian tumor.

The fact that three members of Akt family are down stream targets of PI 3-kinase and are regulated by similar mechanism suggests that AKT1 and AKT3 may also be activated in human ovarian cancer. In this study, we focused on activation of AKT2 in primary ovarian cancer. However, we have shown activation of AKT1 and PI 3-kinase but not AKT2 in five ovarian cancer specimens, which express high levels of AKT1 but very low levels of AKT2 (Figure 3). Additional experiments to evaluate the significance of this finding are in progress.

We have previously demonstrated that an activated Ras significantly activates AKT2. We have also recently documented that farnesyltransferase inhibitor (FTI)-277, an anticancer drug blocking the posttranslational farnesylation of oncogenic Ras, inhibits PI 3-kinase and AKT2 activity and induces apoptosis in

AKT2 overexpressing cell lines (Jiang *et al.*, 2000). Overexpression of AKT2, but not Ras, sensitizes NIH3T3 cells to FTI-277. However, FTI-277 is not a direct inhibitor of PI 3-kinase and AKT2. In this study, we demonstrate that direct inhibition of PI 3-kinase/Akt pathway by wortmannin or LY294002 induces apoptosis in ovarian cancer cell lines with PI 3-kinase/AKT2 activation. Moreover, we have also shown that the majority of tumors with activated AKT2 are high grade and late stage. These data indicate that activation of PI 3-kinase/Akt may play a pivotal role in development of human ovarian cancer, especially in tumor progression, and that PI 3-kinase/Akt pathway could be an important target for intervention of this malignancy. Future studies are required to define the role of Akt activation in ovarian malignant transformation.

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